

Attorney's Docket No. 1847/30

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Anticipated Classification of this application:

Class _____ Subclass _____

Prior application:

Examiner: C. Chin

Art Unit: 1641

Box Patent Application
Commissioner of Patents and Trademarks
Washington, D.C. 20231

TRANSMITTAL OF FILING UNDER 37 CFR 1.60(b)

WARNING: A C-I-P (continuation-in-part) *cannot* be filed under 37 CFR 1.60(b).

WARNING: A filing under 37 C.F.R. § 1.60(b) can only be made if the "prior application was a nonprovisional application and a complete application as set forth in § 1.51(a)(1)." 37 C.F.R. § 1.60(b)(1).

WARNING: Filing under 37 CFR 1.60 is permitted only if filed by the same or less than all the inventors named in the prior application. 37 CFR 1.60(b)(3).

WARNING: The filing of an application at the United States stage of an International Application requires an oath or declaration. 37 CFR 1.61(a)(4).

WARNING: The claims of this new application may be finally rejected in the first Office action where all claims of the new application are drawn to the same invention claimed in the earlier application and would have been properly finally rejected on the grounds or art of record in the next Office action if they had been entered in the earlier application. MPEP § 706.07(b).

This is a request for filing a

☐ Continuation

☒ Divisional

application under 37 CFR 1.60, of pending prior application

Serial No. 08 / 685,329 filed on July 23, 1996
(Date)

CERTIFICATION UNDER 37 CFR 1.10

I hereby certify that this 37 CFR 1.60 request and the documents referred to as attached therein are being deposited with the United States Postal Service on this date 1/7/99 in an envelope as "Express Mail Post Office to Addressee" service under 37 CFR 1.10, Mailing Label Number EL170413488US addressed to the: Commissioner of Patents and Trademarks, Washington, D.C. 20231.

Susan Powell

(type or print name of person mailing paper)

Susan Powell

(Signature of person mailing paper)

NOTE: Each paper or fee filed by "Express Mail" must have the number of the "Express Mail" mailing label placed thereon prior to mailing. (37 CFR 1.10(b)).

WARNING: Certificate of mailing (first class) or facsimile transmission procedures of 37 CFR 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence.

of Burkhard Raguse; Ronald Pace; Lionel King; Vijoleta Braach-Maksvytis; Peter
(Inventor(s)) Osman
for SELF ASSEMBLY OF SENSOR MEMBRANES
(Title of invention)

NOTE: 37 CFR 1.60 permits the omission of a declaration only if the prior application was complete as set forth in 37 CFR 1.51(a), namely, the prior application comprised at least (1) a specification, including a claim or claims; (2) a declaration; (3) drawings when necessary; and (4) the prescribed filing fee. Accordingly, as presently worded, 37 CFR 1.60 does not permit this procedure to be used where the prior application is pending but only the processing and retention fee required by 37 CFR 1.21(f) is paid or where the declaration was not filed.

1. Copy of Prior Application as Filed That is Attached

NOTE: Under 37 CFR 1.60, practice signing and execution of the application by the applicant may be omitted provided the copy is supplied by and accompanied by a statement by the applicant or his or her attorney or agent that the application papers comprise a true copy of the prior application as filed and that no amendments referred to in the declaration filed to complete the prior application introduced new matter therein.

NOTE: This statement need not be verified if made by an attorney registered to practice before the PTO. (37 CFR 1.60(b)).

- ☒ I hereby verify that the attached papers are a true copy of what is shown in my records to be the above identified prior application, including the oath or declaration originally filed. (37 CFR 1.60(b)(2))

The copy of the papers of prior application as filed which are attached are as follows:

- ☒ 23 page(s) of specification
☒ 11 page(s) of claims
☒ 1 page(s) of abstract
☒ 15 sheet(s) of drawing

(also complete part 6 below, if drawings are to be transferred)

- ☒ 3 pages of declaration and power of attorney

(If the copy of the declaration being filed does not show applicant's signature, because the attorney's records do not contain a copy of the signed declaration actually filed for the application, indicate thereon that it was signed and complete the following:)

- ☒ in accordance with the indication required by 37 CFR 60(b), my records reflect that the original signed declaration showing applicant's signature was filed on October 8, 1996

- ☐ the amendment referred to in the declaration filed to complete the prior application and I hereby state, in accordance with the requirements of 37 CFR 1.60(b), that this amendment did not introduce new matter therein.

2. Amendments

WARNING: *"The claim of a new application may be finally rejected in the first Office action in those situations where (1) the new application is a continuing application of, or a substitute for, an earlier application, and (2) all the claims of the new application (a) are drawn to the same invention claimed in the earlier application, and (b) would have been properly finally rejected on the grounds or art of record in the next Office action if they had been entered in the earlier application."* MPEP § 706.07(b).

- ☒ Cancel in this application original claims 1-61 of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)
- ☒ A preliminary amendment is enclosed. (Claims added by this amendment have been properly numbered consecutively beginning with the number next following the highest numbered original claim in the prior application.)

NOTE: *Only amendments reducing the number of claims or adding a reference to the prior application (§ 1.78(a)) will be entered before calculating the filing fee and granting the filing date. 37 CFR 1.60(b)(4).*

NOTE: *"When filing under Rule 1.60 retain at least one original claim from the patent application to assure a complete application." Notice of March 3, 1986 (1064 O.G. 37-38).*

3. Petition for Suspension of Prosecution for the Time Necessary to File an Amendment

NOTE: *Where it is possible that the claims on file will give rise to a first action final for this continuation application and for some reason an amendment cannot be filed promptly (e.g., experimental data is being gathered) it may be desirable to file a petition for suspension of prosecution for the time necessary).*

(check the next item, if applicable)

- ☐ There is provided herewith a Petition To Suspend Prosecution For The Time Necessary to File An Amendment (New Application Filed Concurrently).

4. Information Disclosure Statement

(check this item, if applicable)

- ☐ An information disclosure statement is submitted herewith.

5. Fee Calculation (37 CFR 1.16)

| CLAIMS AS FILED | | | | | | |
|---|--------------|--------|---|------|-----------------------------|----------|
| Number filed | Number Extra | | | Rate | Basic Fee 37 CFR 1.16(a) | |
| | | | | | \$760.00 | |
| Total | | | | | | |
| Claims (37 CFR 1.16(c)) | 21 | - 20 = | 1 | × | \$ 18.00 | \$ 18.00 |
| Independent | | | | | | |
| Claims (37 CFR 1.16(b)) | 4 | - 3 = | 1 | × | \$ 78.00 | \$ 78.00 |
| Multiple dependent claim(s), if any (37 CFR 1.16(d)) | | | | | | |
| | | | | + | | |

☐ Fee for extra claims is not being paid at this time. (37 CFR 1.16(d))

NOTE: If the fees for extra claims are not paid on filing they must be paid or the claims cancelled by amendment, prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency. 37 CFR 1.16(d).

Filing Fee Calculation

\$ 856.00

6. Small Entity Status

☐ A verified statement that this filing is by a small entity:

☐ is attached

☐ has been filed in the parent application and such status is still proper and desired (37 CFR 1.28(a))

Filing Fee Calculation (50% of above) \$

NOTE: Any excess of the full fee paid will be refunded if a verified statement is filed within 2 months of the date of timely payment of a full fee then the excess fee paid will be refunded on request. 37 CFR 1.28(a).

NOTE: 37 CFR 1.28(a), last sentence states: "Applications filed under § 1.60 or § 1.62 of this part must include a reference to a verified statement in a parent application if status as a small entity is still proper and desired."

7. Drawings

☒ Drawings are enclosed

☒ formal

☐ informal

WARNING: DO NOT submit original drawings. A high quality copy of drawings should be supplied when filing a patent application. The drawings that are submitted to the Office must be on strong, white, smooth, and non-shiny paper and meet the standards of § 1.84. If corrections to the drawings are necessary, they should be made to the original drawings and a high-quality copy of the corrected original drawing then submitted to the Office. Only one copy is required or desired. Comments on proposed new 37 CFR 1.84. Notice of March 9, 1988 (1090 O.G. 57-62).

NOTE: "Identifying indicia, if provided, should include the application number or the title of the invention, inventor's name, docket number (if any), and the name and telephone number of a person to call if the Office is unable to match the drawings to the proper application. This information should be placed on the back of each sheet of drawing a minimum distance of 1.5 cm. (5/8 inch) down from the top of the page." 37 C.F.R. 1.84(c)).

8. Priority—35 U.S.C. 119

- ☒ Priority of application Serial No. 0 / PN3669 filed on June 20, 1995 in Australia is claimed under 35 U.S.C. 119. ** (country)
- ☒ The certified copy has been filed in prior U.S. application Serial No. 08 / 685,329 on July 23, 1996.
- ☐ The certified copy will follow.

9. Relate Back—35 U.S.C. 120

- ☒ Amend the specification by inserting, before the first line, the following sentence:
- "This is a
- ☐ continuation
- ☒ divisional
- of copending application(s)
- ☒ Serial number 08 / 685,329 filed on July 23, 1996 "
- ☐ International Application _____ filed on _____ and which designated the U.S."

NOTE: The proper reference to a prior filed PCT application which entered the U.S. national phase is the U.S. serial number and the filing date of the PCT application which designated the U.S.

10. Inventorship Statement

NOTE: "If the continuation or divisional application is filed by less than all the inventors named in the prior application a statement must accompany the application when filed requesting deletion of the names of the person or persons who are not inventors of the invention being claimed in the continuation or divisional application." 37 CFR 1.60(b)(4) [emphasis added].

(complete appropriate items (a) and (b))

- (a) With respect to the prior copending U.S. application from which this application claims benefit under 35 USC 120 the inventor(s) in this application is (are):

(complete applicable item below)

- ☒ the same
- ☐ less than those named in the prior application and it is requested that the following inventor(s) identified above for the prior application be deleted:

(type name(s) of inventor(s) to be deleted)

- (b) The inventorship for all the claims in this application are

- ☒ the same
- ☐ not the same, and an explanation, including the ownership of the various claims at the time the last claimed invention was made, is submitted.

(37 CFR 1.60(b) [4-3]—page 5 of 9)

**** Additional Priority - PCT/AU96/00369; Filed: June 20, 1996**
Country: PCT

11. Assignment

☒ The prior application is assigned of record to Australian Membrane and Biotechnology Research Institute and The University of Sydney

☐ An assignment of the invention to _____

is attached. A separate ☐ "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or ☐ FORM PTO 1595 is also attached.

NOTE: "If an assignment is submitted with a new application, send two separate letters - one for the application and one for the assignment." Notice of May 4, 1990 (1114 O.G. 77-78).

NOTE: When an assignee files a . . . divisional application (under . . . 1.60 . . .) reference may be made to a statement filed under 37 CFR 3.73(b) in the parent application, or a copy of that statement may be filed. Notice of April 30, 1993, 1150 O.G. 62-64.

12. Fee Payment Being Made At This Time

☐ Not Enclosed

☐ No filing fee is submitted. (This and the surcharge required by 37 CFR 1.16(e) can be paid subsequently).

☒ Enclosed

☒ basic filing fee \$ 856.00

☐ recording assignment
(\$40.00; 37 CFR 1.21(h))
(See attached "COVER SHEET FOR ASSIGNMENT ACCOMPANYING NEW PATENT APPLICATION".)

☐ processing and retention fee
(\$130.00; 37 CFR 1.53(d) and 1.21(l)) \$ 856.00

NOTE: 37 CFR 1.21(l) establishes a fee for processing and retaining any application which is abandoned for failing to complete the application pursuant to 37 CFR 1.53(d) and this, as well as the changes to 37 CFR 1.53 and 1.78 indicate that in order to obtain the benefit of a prior U.S. application, either the basic filing fee must be paid or else the processing and retention fee of § 1.21(l) must be paid within 1 year from notification under § 53(d).

Total fees enclosed \$ 856.00

13. Method of Payment of Fees

☒ Enclosed is a check in the amount of \$ 856.00

☐ Charge Account No. 07-1730 in the amount of \$ any deficiency
A duplicate of this request is attached.

NOTE: Fees should be itemized in such a manner that is clear for which purpose the fees are paid. 37 CFR 1.22(b).

14. Authorization To Charge Additional Fees

WARNING: If no fees are being paid on filing do not complete this item.

WARNING: Accurately count claims, especially multiple dependent claims, to avoid unexpected high charges if extra claim charges are authorized.

☐ The Commissioner is hereby authorized to charge the following additional fees which may be required by this paper and during the entire pendency of the application to Account No. _____.

☐ 37 CFR 1.16 (a), (f) or (g) (filing fees)

☐ 37 CFR 1.16 (b), (c) and (d) (presentation of extra claims)

NOTE: Because additional fees for excess or multiple dependent claims not paid on filing or on later presentation must only be paid or these claims cancelled by amendment prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency (37 CFR 1.16(d)) it might be best not to authorize the PTO to charge additional claim fees, except possibly when dealing with amendments after final action.

☐ 37 CFR 1.17 (application processing fees)

WARNING: While 37 CFR 1.17(a), (b), (c) and (d) deal with extensions of time under § 1.136(a) this authorization should be made only with the knowledge that: "Submission of the appropriate extension fee under 37 CFR 1.136(a) is to no avail unless a request or petition for extension is filed." [emphasis added]. Notice of November 5, 1985 (1060 O.G. 27).

☐ 37 CFR 1.18 (issue fee at or before mailing Notice of Allowance, pursuant to 37 CFR 1.311(b)).

NOTE: Where an authorization to charge the issue fee to a deposit account has been filed before the mailing of a Notice of Allowance, the issue fee will be automatically charged to the deposit account at the time of mailing the notice of allowance. 37 CFR 1.311(b)).

NOTE: 37 CFR 1.28(b) requires "Notification of any change in status resulting in loss of entitlement to small entity status must be filed in the application . . . prior to paying or at the time of paying . . . issue fee." From the wording of 37 CFR 1.28(b): (a) notification of change of status must be made even if the fee is paid as "other than a small entity" and (b) no notification is required if the change is to another small entity.

15. Power of Attorney

☒ The power of attorney in the prior application is to

Allen I. Rubenstein, Esq.

27,673

(Attorney)

(Reg. No.)

- a. ☒ The power appears in the original papers in the prior application.
- b. ☐ Since the power does not appear in the original papers, a copy of the power in the prior application is enclosed.
- c. ☐ A new power has been executed and is attached.
- d. ☐ Address all future communications to

(item d may only be completed by applicant, or attorney or agent of record)

Allen I. Rubenstein, Esq.

GOTTLIEB, RACKMAN & REISMAN, P.C.

270 Madison Avenue, 8th Floor

New York, New York 10016

16. Maintenance of Copendency of Prior Application

(this item must be completed and the papers filed in the prior application if the period set in the prior application has run.)

- ☐ A petition, fee and response has been filed to extend the term in the pending prior application until _____ .

NOTE: The PTO finds it useful if a copy of the petition filed in the prior application extending the term for response is filed with the papers constituting the filing of the Continuation Application. Notice of November 5, 1985 (1060 O.G. 27).

- ☐ A copy of the petition for extension of time in the prior application is attached.

17. Conditional Petition for Extension of Time in Prior Application

(complete this item and file conditional petition in the prior application if previous item not applicable)

- ☐ A conditional petition for extension of time is being filed in the pending parent application.

NOTE: The PTO finds it useful if a copy of the petition filed in the prior application extending the term for response is filed with the paper constituting the filing of the continuation application. Notice of November 5, 1985 (1060 O.G. 27).

- ☐ A copy of the conditional petition for extension of time in the prior application is attached.

18. Abandonment of Prior Application *(if applicable)*

WARNING: *(Do not complete this item if the application being filed is a divisional of the prior application which is not being abandoned).*

NOTE: "A registered attorney or agent acting under the provisions of § 1.34(a), or of record, may also expressly abandon a prior application as of the filing date granted to a continuing application when filing such a continuing application." 37 CFR 1.138.

- ☐ Please abandon the prior application at a time while the prior application is pending or when the petition for extension of time or to revive in that application is granted and when this application is granted a filing date so as to make this application copending with said prior application.

19. Notification in Parent Application of the Filing of This Continuation Application

- ☐ A notification of the filing of this continuation is being filed in the parent application from which this application claims priority under 35 USC § 120.

20. Statement by Assignee (if applicable)

- ☐ In accordance with 37 CFR 3.73, I have reviewed the evidentiary documents establishing my/our ownership of the application identified herein, and certify that to the best of my/our knowledge and belief, title is with me/us who seek to take action.

☐ Assignment submitted herewith for recordal

I hereby declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Allen I. Rubenstein

(type or print name of person signing
declaration)

Allen I. Rubenstein
Signature

1/7/95
Date

Gottlieb, Rackman & Reisman, P.C.
(P.O. Address of Signatory)

270 Madison Avenue 8th Floor
New York, New York 10016

Tel. No. : (212) 684-3900
Reg. No. 27,673
(if applicable)

- ☐ Inventor
☐ Assignee of complete interest
☐ Person authorized to sign on behalf of assignee
☒ Attorney or agent of record
☐ Filed under Rule 34(a)

(complete the following if applicable)

(Type name of assignee)

(Title of person authorized to sign on behalf
of assignee)

(Address of assignee)

Assignment recorded in PTO on

Reel _____

Frame _____

The statement under 37 CFR 3.73(b)

- ☐ has been filed in the parent application.
☐ a copy of the statement previously filed in the parent application is attached.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Raguse et al.
Examiner : C. Chin
Title : SELF ASSEMBLY OF SENSOR MEMBRANES

PRELIMINARY AMENDMENT

Assistant Commissioner for
Patents
Washington, DC 20231

Dear Sir/Madam:

Prior to substantive examination of the subject application, kindly amend the claims as follows:

Cancel claims 1-61.

In claim 67, change "62 or 63" to -- 62 --.

In claim 72, change "any one of claims 69 to 71" to -- claim 69 --.

In claim 73, change "any one of claims 69 to 72" to -- claim 69 --.

Please amend the following claims:

81. (Amended) A method [according to any one of claims 13 to 21] of producing a monolayer electrode membrane comprising:

- (1) Forming a solution containing the disulfide of mercaptoacetic acid (MAAD) or 2-mercaptoethanol (EDS), membrane spanning lipid C

(MSL-C) and/or membrane spanning lipid D (MSL-D) and, optionally, Linker Lipid A, linker Gramicidin B;

- (2) Contacting an electrode containing a clean gold surface with the solution, the disulfide of mercaptoacetic acid (MAAD) or 2-mercaptoethanol (EDS) in the solution thus adsorbing onto the gold surface of the electrode;
- (3) Rinsing the electrode with a suitable organic solvent; and
- (4) Removing the excess organic solvent used for rinsing, wherein valinomycin is covalently linked to the MSL-C or MSL-D via a linker of appropriate length to permit the valinomycin to diffuse from one side of the membrane to another.

82. (Amended) A method [according to claim 1 of 2] of producing a first layer electrode membrane comprising:

- (1) Forming a solution containing Linker Lipid A, the disulfide of mercaptoacetic acid (MAAD) or 2-mercaptoethanol (EDS), linker Gramicidin B, membrane spanning lipid C (MSL-C) and membrane spanning lipid D (MSL-D) wherein the ratio of Linker Lipid A to the disulfide of mercaptoacetic acid (MAAD) or 2-mercaptoethanol (EDS) is 2:1, the ratio of Linker Lipid A + MAAD or EDS to MSL-D is in the range of 10:1 to 100:1, and the ratio of Linker Lipid A + MAAD or EDS to MSL-C is between 20,000:1 and 100:1;

- (2) Contacting an electrode containing a clean gold surface with the solution, the disulfide containing components in the solution thus adsorbing onto the gold surface of the electrode;
- (3) Rinsing the electrode with a suitable organic solvent; and
- (4) Removing the excess organic solvent used for rinsing, wherein the ratio of Lipid Linker A to the disulfide of mercaptoacetic acid (MAAD) or 2-mercaptoethanol (EDS) is in the range of 5:1 to 1:2.

REMARKS

By this Amendment, multiple dependency of the remaining claims is removed, and the dependence from cancelled claims is avoided by incorporating the limitations of the parent claims.

Claim 81 depended from cancelled claim 13. Claim 13 had been amended during the prosecution of the allowed claims of the parent application and those amendments have been included into claim 81.

Claim 82 depended from cancelled claim 1. Claim 1 had been amended during the prosecution of the allowed claims of the parent application and those amendments have been included into claim 82.

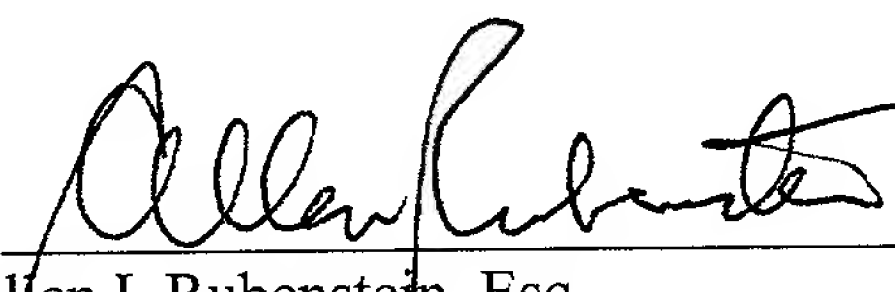
Each of the foregoing amendments have been made to avoid objections under 35 U.S.C. §112 and not to overcome or anticipate any objections based upon the prior art.

Enclosed is a check payable to the Commissioner for \$856.00 to cover the filing fee. The Examiner is authorized to charge any additional amount necessitated by this filing to

Deposit Account No. 07-1730, if necessary. Furthermore, throughout the prosecution of this case, the Examiner is authorized to charge Deposit Account No. 07-1730 for any fees for extension of time necessary to allow the prosecution of the application to continue. The Applicants hereby request the granting of such extensions whenever necessary.

Respectfully submitted,

GOTTLIEB, RACKMAN & REISMAN
Attorneys for Applicants
270 Madison Avenue, 8th Floor
New York, New York 10016
(212) 684-3900

By: 
Allen I. Rubenstein, Esq.
Reg. No. 27,673

Dated: New York, New York
January 7, 1999

SELF ASSEMBLY OF SENSOR MEMBRANES

5 The present invention relates to electrode membrane combinations for use in biosensors to detect analytes in a sample and to methods for the production of such electrode membrane combinations. The present invention also relates to methods for storing such electrode membrane combinations.

10 Biosensors based on ion channels or ionophores contained within lipid membranes that are deposited onto metal electrodes and where the ion channels are switched in the presence of analyte molecules have been described in International patent specification Nos WO 92/17788, WO 93/21528, WO 94/07593 and U.S. 5,204,239 (the disclosures of which are incorporated herein by reference) . As is disclosed in these applications, ionophores such as gramicidin ion channels may be co-dispersed with
15 amphiphilic molecules, thereby forming lipid membranes with altered properties in relation to the permeability of ions. There is also disclosure of various methods of gating these ion channels (for example, the lateral segregation mechanism disclosed in International Patent Application W090/08783) such that in response to the binding of an analyte to a binding
20 partner attached to the membrane, the conductivity of the membrane is altered. The applications also disclose methods of producing membranes with improved sensitivity using a surface amplifier effect, and improved stability and ion flux using chemisorbed arrays of amphiphilic molecules attached to an electrode surface. The applications further disclose means of
25 producing lipid membranes incorporating ionophores on said chemisorbed amphiphilic molecules.

The present inventors have now determined improved means of producing electrode membrane combinations that result in sensor membranes with improved properties in terms of reproducibility, gating
30 response towards an analyte, lateral segregation response, surface amplifier effect, stability in serum, plasma and blood, simplified production and the ability to store the membranes in a dry format (i.e. in the absence of any aqueous bath solution).

In the first aspect, the present invention consists in a method of
35 producing a first layer electrode membrane comprising:-

- (1) Forming a solution containing Linker Lipid A (figure 1) , the disulfide of mercaptoacetic acid (MAAD) or similar molecule, such as EDS linker Gramicidin B (figure 2), membrane spanning lipid C (MSL-C) (figure 3) and membrane spanning lipid D (MSL-D) (figure 3) or other linker molecules and ion channel or ionophore combinations as previously described;
- (2) Contacting an electrode containing a clean gold surface with the solution, the disulfide containing components in the solution thus adsorbing onto the gold surface of the electrode;
- (3) Rinsing the electrode with a suitable organic solvent; and
- (4) Removing the excess organic solvent used for rinsing.

The nature of the membrane components are as follows:-

Linker Lipid A comprising a benzyl disulfide attachment region, a hydrophilic region composed, in sequence, of tetraethylene glycol, succinic acid, tetraethylene glycol and succinic acid subgroups and an aliphatic chain;

The disulfide of mercaptoacetic acid (MAAD) or similar molecule, such as the disulfide of 2-mercaptoethanol (EDS).

Linker Gramicidin B is a linker molecule which comprises a benzyl disulfide attachment region, a hydrophilic region composed, in sequence, of tetraethylene glycol, succinic acid, tetraethylene glycol, succinic acid, and a hydrophobic region of gramicidin;

Membrane spanning lipid (MSL) D which comprises a benzyl disulfide attachment region, a hydrophilic region composed, in sequence, of tetraethylene glycol, succinic acid, tetraethylene glycol, succinic acid and a hydrophobic region of 1,1'-dotriacontamethylenebis (2-3 RS,7R, 11- phytanyl) with an intermediate biphenyl region and a head group of phosphatidylcholine, hydroxyl, succinic acid, or PEG-400 COOH; and

Membrane spanning lipid C which comprises the same attachment and hydrophilic region as membrane spanning lipid D but differs in the head group which is a group consisting of (one to eight) 1,6-amino caproic acid and biotin.

In a preferred embodiment of the present invention the ratio of Linker Lipid A to the disulfide of mercaptoacetic acid (MAAD) or 2-mercaptoethoethanol (EDS) is 5:1 to 1:2, more preferably is 2:1.

It is further preferred that in order to improve the stability of the membrane, the amount of MSL- D in the first layer is as high as can be allowed and still maintain reasonable gramicidin conduction. The ratio of (Linker Lipid A + MAAD or EDS) to MSL-D is therefore preferably between 10:1 to 100:1.

In a further preferred embodiment, the amount of MSL-C is such that in the final sensor membrane an effective surface amplification on addition of analyte occurs, while still making it possible to suppress the lateral segregation induced gating on addition of the streptavidin, avidin or other similar biotin-binding protein. It should be noted that if the amount of MSL-C in the final sensor membrane is too large, then the excess protein that is bound to the MSL-C on addition of the streptavidin, avidin or similar biotin-binding protein will restrict the mobility of the gramicidin/receptor couple thereby reducing the gating response. In cases where the analyte molecule has multiple identical epitopes, MSL-C may capture the analyte molecules in preference to gramicidin/receptor couple, reducing the biosensor response.

It is therefore preferred that the ratio of (Linker Lipid A + MAAD or EDS) to membrane spanning lipid C is between 20,000:1 and 100:1.

It is further preferred that the ratio of (Linker Lipid A + MAAD or EDS) to MSL-C is 20,000:1.

As is known in the art, gramicidin exists in a monomer/dimer equilibrium in a bilayer membrane. In order for the gramicidin lateral segregation switch to function effectively, the ratio of monomer to dimer must be controlled. It is preferred that a proportion of the gramicidin ion channels exist as freely diffusing monomers in the outer membrane layer. The ratio of monomers to dimers can be controlled, amongst other methods, by changing the concentration of gramicidin in the first and second half of the membrane.

It is therefore preferred that the ratio of (Linker Lipid A + MAAD or EDS) to linker Gramicidin B is 10,000:1.

It is further preferred that the ratio of (Linker Lipid A + MAAD or EDS) to linker Gramicidin B is between 20,000:1 and 100,000:1 in those cases where it is necessary to minimise the amount of background leakage due to the adsorbed linker Gramicidin B.

It is preferred that the gold electrode consists of a freshly evaporated or sputtered gold electrode. It is further preferred that the gold electrode surface be freshly cleaned using a plasma etching process or an ion beam milling process.

5 It is preferred that the solvent for the adsorbing solution (step (1) and for the rinsing step (4) is ethanol.

In a second aspect, the present invention consists in a method of producing a monolayer electrode membrane comprising:-

- 10 (1) Forming a solution containing the disulfide of mercaptoacetic acid (MAAD) or similar molecule (e.g. 2-mercaptoethanol (EDS)), membrane spanning lipid C(MSL-C) and/or membrane spanning lipid D (MSL-D) and, optionally, Linker Lipid A, linker Gramicidin B or other linker molecules or ion channel or ionophore combinations;
- 15 (2) Contacting an electrode containing a clean gold surface with the solution, the disulfide containing components in a solution thus adsorbing onto the gold surface of the electrode;
- (3) Rinsing the electrode with a suitable organic solvent; and
- (4) Removing the excess organic solvent used for rinsing,
- 20 wherein the solution in step (1) contains more than a molar % of 50% of a membrane spanning lipid.

More preferably, the solution in step (1) contains more than a molar % or 70% of a membrane spanning lipid, 29% MAAD or EDS and 1% other membrane spanning lipids.

25 The preferred features and embodiments discussed above in regard to the method of the first aspect of the invention, may be equally applicable to the method of the second aspect of the invention.

The membranes produced by the method of the second aspect of the invention, do not form bilayers and have been found to be particularly resistant towards non-specific effects on addition of serum, plasma or whole blood to the sensor. Further advantages have been noted in that these
 30 membranes may be reused over a period of months in serum, plasma or whole blood without showing signs of degradation of performance. Monolayer lipid membranes are more practical for manufacturing purposes, have fewer manufacturing steps and greater stability, leading to a later
 35 expiry on the manufactured sensor containing such membranes. In this it is also preferable for the spacer molecule, MAAD or EDS, to be covalently

linked to the membrane spanning lipids C or D, and covalently linked to PEPC, GDPE or triphytanyl PC, which increases stability of the final membrane.

5 A further preferred embodiment of the method according to the second aspect of the invention, consists in the use of valinomycin, covalently linked to the membrane spanning lipids C or D, via a linker of appropriate length such that the valinomycin is able to diffuse from one side of the membrane to another. This then results in a reusable biosensor, which does not need replenishment of the ionophore and could be used for
10 an implantable device.

The present inventors have determined that the production of the biosensor is simplified and improved through the use of streptavidin, avidin or one of the related biotin binding - proteins as a means of coupling a biotinylated receptor onto a biotinylated gramicidin ion channel or MSL.

15 In a third aspect, the present invention consists in a method of producing a second layer electrode membrane combination utilising biotinylated gramicidin E, in which the biotin is attached to the gramicidin via an amide to a lysine residue (preferred for chemical stability) or via an ester link to ethanolamine using a linker arm that is made up of between 1
20 to 8 aminocaproyl groups. The linker length, type, valency and number of linkers can affect the stability of the completed sensor and the optimum linker varies depending on the analyte being measured. The method comprises:

- 25 (1) Adding a solution of lipid and biotinylated gramicidin E (figure 4) , dispersed in a suitable solvent onto the electrode surface containing a first layer produced as described in the first aspect of the present invention;
- (2) Rinsing the electrode surface with an aqueous solution;
- (3) Adding a solution of streptavidin, avidin, neutravidin, avidin or
30 streptavidin derivative;
- (4) Rinsing the electrode with an aqueous solution in order to remove excess streptavidin, avidin, neutravidin or other avidin or streptavidin derivative;
- (5) Adding a solution of a biotinylated binding partner molecule: and
35 (6) Rinsing the coated electrode with an aqueous solution.

In a preferred embodiment of the present invention the lipid used in

step (1) of the method of the third aspect is a mixture of diphytanyl phosphatidyl choline and glyceryl diphytanyl ether. The inventors have found that the combination of these lipids improves the stability of the bilayer membrane towards serum, plasma and whole blood, while still maintaining a good ionic seal, fluidity, reducing temperature effects on conduction and maintaining a true bilayer membrane structure.

It is further preferred that the diphytanyl phosphatidyl choline (DPEPC) and glyceryl diphytanyl ether (GDPE) is in a 7:3 ratio.

It is further preferred that the lipid is a triphytanyl phosphoryl choline as shown in figure (6).

It is also preferred that membranes contain 0 to 50%, more preferably 0 to 20% cholesterol in the second layer to enhance stability and analyte response in a serum, plasma or whole blood sample.

It is preferred that the ratio of lipid to biotinylated gramicidin E is between 10,000:1 and 1,000,000:1.

It is further preferred that the ratio of lipid to biotinylated gramicidin E is 100,000: 1.

It is preferred that the biotin is attached to the gramicidin via the ethanolamine end using a linker arm that is between 10-80 angstroms long. It is preferred that the linker arm is hydrophilic.

It is preferred that the biotin is attached to the gramicidin via the ethanolamine end using a linker arm that is made up of between 1 to 8 aminocaproyl groups.

It is further preferred that two biotins are attached to the gramicidin via the ethanolamine end such that the biotins are able to bind simultaneously into the adjacent binding sites of one streptavidin, avidin or similar biotin-binding protein molecule, or into two separate streptavidin avidin or similar biotin-binding protein molecules.

Alternatively, more than two biotin molecules can be attached to the gramicidin to produce multiple attachment sites for the binding partner molecules.

It is preferred that the two biotins are attached to the gramicidin via the ethanolamine end such that each biotin is attached to two to four linearly joined aminocaproyl groups that are attached to a lysine group as shown in figure (5). When more than two biotin molecules are attached to

the gramicidin, a longer linker up to twenty aminocaproyl groups may be necessary these may be organised linearly or as a branched structure.

It is further preferred that in order to optimise the analyte response, it is necessary to minimise the signal caused by the presence of the linker.

5 Thus, the amount of streptavidin, avidin or other similar biotin-binding protein that is added in step (3) is sufficient to cause a prozone effect, allowing most of the available biotinylated species in the membrane to have one streptavidin or related molecule bound to prevent crosslinking between gramicidin channels and MSL until a sample containing analyte is added to
10 the sensor.

It is further preferred that prior to the addition of the streptavidin, avidin, or similar biotin-binding protein the lipid membrane electrode assembly is cooled. This reduces the fluidity of the membrane, decreasing the mobility of membrane components thus allowing the streptavidin, avidin
15 or other similar biotin-binding protein to more readily bind to the biotinylated Gramicidin E and the membrane spanning lipid C without crosslinking between gramicidin channels and MSL until a sample containing analyte is added to the sensor.

It is preferred that the lipid membrane electrode is cooled to between
20 0° and 50°C, more preferably 0° and 5°C. It is further preferred that the subsequent rinsing and addition of the biotinylated binding partner molecule are also carried out at 0° to 50°C, more preferably 0° to 5°C.

It is preferred that the binding partner molecule is a biotinylated antibody or biotinylated antibody fragment.

25 It is further preferred that the binding partner molecule is a Fab' fragment that is biotinylated via the free Fab' thiol group.

Is further preferred that the linker between the Fab' and biotins is between 10-80 angstroms in length. Is further preferred that the linker between the Fab' and biotins consists of one to eight aminocaproyl groups.

30 It is further preferred that the group containing two biotins is attached to the antibody or antibody fragment such that the two biotins are able to complex simultaneously one streptavidin, avidin or other similar biotin-binding protein or two adjacent streptavidin, avidin or other similar biotin-binding protein molecules

35 Alternatively, more than two biotins may be attached to the antibody or antibody fragment.

Furthermore, the present inventors have determined that by producing a covalently or passively coupled conjugate between the binding partner molecule and the streptavidin, avidin or other similar biotin binding protein the production of the biosensor membrane is further simplified.

5 Accordingly, steps 3 to 5 of the method of the third aspect can be substituted with:

(3) Adding a solution containing a conjugate between streptavidin, avidin, neutravidin or other avidin or streptavidin derivative and a molecule which is a member of a binding pair.

10 It is preferred that the binding partner molecule is an antibody or an antibody fragment such as an Fab or Fab' or Fv fragment. Other binding pairs, which could be used in this invention would include: naturally occurring binding proteins and cellular receptors/analytes, enzymes or enzyme analogues/substrates, lectins/carbohydrates, complementary nucleic acid sequences and Anti-FC, Protein A or Protein G/antibody.

15 In order to manufacture sensor membranes efficiently and reproducibly, it is advantageous to incorporate the ionophore separate to the assembled membrane. It is also advantageous to bind one binding partner to the ionophore, before incorporation into the membrane. This both controls and enhances the reproducibility of membrane conduction and allows the reproducible attachment of the second binding partner needed in a two site immuno- or similar assay system, ensuring that only the first binding partner is attached to ionophore and only the second binding partner is attached to a second ionophore or MSL.

25 The present inventors have found that it is possible to co-disperse the hydrophobic ionophore in aqueous solution by several means, including:

1. The presence of a detergent, preferably at levels below the critical micelle concentration of the detergent, such that ionophore and the detergent form aggregates which allow the ionophore to remain in solution;
2. Conjugation of the gramicidin or other ionophore to a large molecular weight water soluble species; and
3. Attachment of the ionophore to a bead.

35 Furthermore it was found that it was possible to incorporate the functional ionophore into the biosensor lipid membranes by adding an aqueous solution of the ionophore/detergent aggregate to the solution

bathing the preformed lipid biosensor membrane. This method of addition of the ionophore allows for a more controlled and reproducible method of incorporation of the ionophore into the lipid membrane.

Accordingly, in a fourth aspect, the present invention consists in a method of producing a second layer electrode membrane combination comprising:-

- (1) Adding a solution of lipid dispersed in a suitable solvent onto the electrode surface containing a first layer produced as described in the method of the first aspect of the present invention;
- (2) Rinsing the electrode surface with an aqueous solution;
- (3) Adding an aqueous solution containing ionophore co-dispersed with detergent or solubilised by coupling to a high molecular weight soluble species;
- (4) Rinsing the electrode with an aqueous solution; and
- (5) Adding the receptor using either streptavidin, avidin, or other similar biotin-binding protein followed by addition of a biotinylated antibody or antibody fragment or adding a streptavidin, avidin or similar biotin-binding protein conjugated to an antibody or antibody fragment as detailed in the third aspect of the present invention.

In a preferred embodiment of the present invention the lipid used in step (1) is a mixture of diphytanyl phosphatidyl choline and glyceryl diphytanyl ether. It has been found that the combination of these lipids improves the stability of the bilayer membrane towards serum, plasma and whole blood, while still maintaining a good ionic seal, fluidity, reducing temperature effects on conduction and maintaining a true bilayer membrane structure.

It is further preferred that the diphytanyl phosphatidyl choline and glyceryl diphytanyl ether is in a 7:3 ratio.

It is further preferred that the lipid is a triphytanyl phosphoryl choline as shown in figure (6).

It is also preferred that membranes contain 0 to 50%, more preferably up to 20% cholesterol in the second layer to enhance stability and analyte response in a serum, plasma or whole blood sample.

It is preferred that the aqueous solution used in step (3) of the fourth aspect of the present invention, contains gramicidin or a gramicidin derivative that is added to an aqueous solution of a detergent such that the

detergent is present in excess relative to the gramicidin but that the total concentration of detergent is below the critical micelle concentration (CMC). The total detergent concentration is preferably kept below the CMC in order to minimise or negate any possible disruption of the membrane by the detergent.

The gramicidin/detergent solution is then preferably sonicated using an ultrasonic bath or horn for 5 to 20 minutes.

Preferred detergents are sodium dodecylsulfate, octylglucoside, tween, or other ionic or non-ionic detergents.

It is further preferred that the alkyl chain contains at least 7 or more methylene groups.

It is preferred that the detergent is sodium dodecylsulfate.

It is further preferred that the concentration of the sodium dodecylsulfate is less than 0.00001M and that the concentration of gramicidin is ten times less than the sodium dodecylsulfate concentration.

It has been found that, if it is necessary to store the electrodes which already have the first layer of the membrane adsorbed onto the electrode surface, then it is advantageous to store said electrodes covered in a solvent. This method of storing the electrode with the first layer membrane in a solution has been found to produce subsequent sensor membranes with improved homogeneity and ionophore gating ability, compared with storing the electrode in air.

Accordingly, in a fifth aspect the present invention consists in a first layer membrane electrode combination comprising an electrode and a first layer membrane comprising a closely packed array of amphiphilic molecules and a plurality of ionophores, the first layer membrane being connected to the electrode by means of a linker group as described previously, said first layer membrane being stored in the presence of a solvent.

Electrodes may be stored in a gaseous or liquid environment and, in a preferred embodiment, the solvent in which the electrodes are stored is an organic solvent or an aqueous solvent.

If the solvent is an organic solvent, it is further preferred that the solvent is an alcohol such as ethanol, glycerol, ethylene glycol, an alcohol or diol containing between 3 to 12 carbon atoms.

It is further preferred that the solvent is a hydrocarbon with between 8 to 20 carbon atoms. It is further preferred that the solvent is an aqueous solution containing a detergent.

5 It is further preferred that the solvent is a compound that is able to coat the electrodes such that oxidation of the electrode surface is minimised. It is preferred that such a solvent can be applied as a thin film.

10 Additionally it has been found that it is possible to store the complete sensor membrane electrode combination in a non-aqueous format. This is highly advantageous in terms of ease of manufacturing, shipping and storing of the biosensor product.

15 Accordingly, in a sixth aspect, the present invention consists in a lipid membrane based biosensor comprising a lipid membrane incorporating ionophores, the conductivity of the membrane being dependent on the presence or absence of an analyte, wherein the aqueous bathing solution in which the biosensor normally resides, is removed in a manner such that, on drying of said lipid membrane biosensor, the lipid membrane and the receptor molecules retain their function, structure and activity, when rehydrated.

20 It is preferred that in the drying process that the biosensor membrane does not have contact with the air-water interface, hence methods of drying such as lyophilisation, evaporation, or evaporation over controlled humidity, are recommended. It is also preferred that the concentration of the water-replacing agent is sufficient to protect all components within the membrane, i.e. lipid, ionophore and protein, during the drying process, during the storage time, and yet is easily removed upon the first addition of analyte or sample in the appropriate matrix, such that full activity of the biosensor membrane is restored immediately.

25 The water replacing substance may be either a protein, a low molecular weight diol or triol, a polyethylene glycol, a low molecular weight sugar, a polymeric peptide, polyelectrolyte or combinations of these substances, all of which are well known in the art. The main attributes of the water substitute are that it is highly polar, has a low vapour pressure, allows the membrane to retain its structure, is protein compatible and does not impede biosensor function when rehydrated. These substances may also be covalently bound to a specific membrane component, preferably a membrane spanning lipid.

35

It is preferred that the water replacing substance is bovine serum albumin, serum, fish gelatin, non-fat dry milk powder, casein, glycerol, ethylene glycol, diethylene glycol, polyethylene glycol, trehalose, xylose, glucose, sucrose, dextrose, ficoll and it is further preferred that the water replacing molecule is glycerol, sucrose, dextran or trehalose.

Such classes of molecules may also have the additional advantage in the biosensor to act as a spreading layer for serum/blood/analyte fluid addition; as a filter against specific cells, bacteria, virus particles, or classes of molecules; or as a reservoir containing specific displacement reagents required to compete off small analytes from proteins to which they are bound in serum or blood.

A further advantage of the water substituting agent is that it allows for the controlled rehydration of the lipid membrane without the lipid bilayer being in contact with the air/water interface as the analyte solution or sample is added.

An example of the latter is given in figure 13, where water-replacing molecules are either added or covalently bound to regions of the membrane and contain, for example, ANS (8-anilino-1-naphthalene-sulfonic acid) which competes with thyroxine for binding sites in albumin and thyroxine-binding globulin (TBG), releasing thyroxine for subsequent detection by the biosensor membrane."

The invention is hereinafter further described with reference to the following non-limiting examples and accompanying figures.

Brief Description of the Figures

Figure 1 shows Linker Lipid A which comprises a disulfide attachment region, a hydrophilic region composed (in sequence) of tetraethylene glycol, succinic acid, tetraethylene glycol, succinic acid subgroups and an aliphatic chain.

Figure 2 shows a linker molecule which comprises a benzyl disulfide attachment region, a hydrophilic region composed (in sequence) of tetraethylene glycol, succinic acid, tetraethylene glycol, succinic acid, and a hydrophobic region of gramicidin.

Figure 3 shows membrane spanning lipids C and D. Membrane spanning lipid C terminates in a group consisting of between one to eight 1,6-amino caproic groups and biotin. Membrane spanning lipid D comprises

the head group which is a group consisting of phosphatidylcholine (PC), OH, succinic acid or polyethylene glycol (PEG) 400.

Figure 4 shows biotinylated gramicidin E in which the biotin is attached to the gramicidin via the ethanolamine end using a linker arm that is made up of between 1 to 8 aminocaproyl groups.

Figure 5 shows two biotins attached to gramicidin via the ethanolamine end with each biotin attached to two to four linearly joined aminocaproyl groups that are attached to a lysine group.

Figure 6 shows a lipid consisting of a triphytanyl phosphoryl choline.

Figure 7 shows the impedance change of the biosensor owing to K⁺ in human blood.

Figure 8 shows repeated whole blood K⁺ detection in monolayer biosensor membrane.

Figure 9 shows that the biosensor response in whole blood was a function of the K⁺ concentration.

Figure 10 shows the detection of ferritin in whole blood and serum.

Figure 11 shows the response of the biosensor to serum concentrations of ferritin.

Figure 12 shows the dependence of the streptavidin response on linker type and length.

Figure 13 shows a network of water-replacing agent (i.e. protectant substance) linked to the biosensor membrane, containing thyroxine displacement reagent such as ANS. As the ANS molecules compete for protein binding sites, thyroxine is released and diffuses to the membrane where it is detected by competitive assay (thyroxine-gramicidin bound to anti-thyroxine Ab/Fab which is bound to membrane spanning lipids, is competed off with the released thyroxine, leading to the release of gramicidin channels and return of ionic conduction in the biosensor).

Figure 14 shows the impedance spectra of a membrane prior to addition of "linker gramicidin E"/SDS (1); after the addition of "linker gramicidin E"/SDS and after addition of streptavidin and biotinylated anti-ferritin Fab' (2); and the spectrum after the addition of ferritin (3).

Figure 15 shows the impedance spectra of a membrane prior to addition of "linker gramicidin F"/streptavidin (1); after the addition of "linker

gramicidin F"/streptavidin and after the addition of biotinylated anti-TSH Fab's (2); and the spectrum after the addition of TSH (3).

5

EXAMPLE 1: Whole blood K⁺ detection in monolayer biosensor membranes

10 Ion carriers other than gramicidin may be used in the membranes described in this invention. Simplified membranes formed mainly from membrane spanning lipid components can form extremely robust and biocompatible biosensors. Fewer membrane components also provides easier manufacturability.

15

Monolayer membrane: 100uM MSLPC (MSL-D)
20uM MAAD

20 Electrodes with freshly evaporated gold (1000A) on chrome adhesion layer (200A on glass microscope slides) were dipped into an ethanol solution of the above components, rinsed with ethanol, then stored at 4°C under ethanol until used for impedance measurements (days-weeks). The slide was clamped into a blocked containing teflon coated wells which defined the area of the working electrode as approximately 16mm². PBS buffer was
25 added followed by 5ul of 1mM valinomycin in ethanol, an ionophore specific for K⁺ detection. Impedance measurements were carried out.

Figure 7 shows that the impedance change showing the biosensor response to K⁺ in human whole blood (obtained from a healthy volunteer) was specific to the presence of valinomycin incorporated into the biosensor
30 membrane. The volume of PBS buffer in the electrode well was completely exchanged for 100ul whole blood which resulted in the decrease in impedance as K⁺ ions in blood were transported across the biosensor membrane by valinomycin. The addition of whole blood showed no detrimental effects to the lipid membrane.

35

Even more convenient assembly of the membrane would be with membrane components which are covalently linked together and

synthesised as a single molecule. Thus a membrane spanning lipid can be linked to spacer molecules such as MAAD or its analogues (to provide correct packing of the first layer which enables essential ion channel or ionophore diffusion), and to extra hydrocarbon chains such as phytanyls to provide increased fluidizing properties to the membrane spanning lipid.

EXAMPLE 2: Repeated whole blood K⁺ detection in same monolayer biosensor membrane

The robust and biocompatible characteristics of the monolayer biosensor can be illustrated by its ability to sustain repeated exposure to whole blood and still function as a biosensor.

Monolayer membrane: 100uM MSL-succinic acid (MSL-D)
20uM MAAD

Electrodes were prepared and measured as described in Example 1. After addition of valinomycin, 80ul PBS buffer was removed from the electrode well (160ul initial volume) and replaced by 80ul whole blood. After the change in impedance due to the K⁺ response was recorded, the well was washed 4 times with PBS buffer and valinomycin was again added to the well before the next exposure of whole blood. The cycle was repeated 4 times as shown in Figure 8, showing the biosensor remaining intact and functioning even after repeated exposure to whole blood.

EXAMPLE 3: Whole blood K⁺ titration in biosensor membranes

This example shows that the detection ability of the biosensor is within the clinically-relevant range of 3-6mM, and above, if required. The biosensor is also stable to whole blood addition even in the bilayer, rather than monolayer, configuration.

1st layer: 100uM MSL - PEG 400 - COOH (MSL-D)
0.8mM MAAD
1mM DLP

2nd layer: 14mM (C18DPEPC:C₁₈GMPE=7:3) : valinomycin = 100:1

(Note: C_{18} DPEPC = DPEPC with 2 additional CH_2 groups; C_{18} GMPE = GDPE with a monophytanyl chain instead of diphytanyl chains and 2 additional CH_2 groups).

5

Electrodes were prepared as described in Example 1. The 2nd layer was added from an ethanolic solution then PBS buffer was added, and the electrode well was washed 4 times. Whole blood was added to different electrodes and the change of impedance with K^+ concentration recorded. Figure 9 shows that the biosensor response to K^+ in human whole blood was a function of the concentration of K^+ in the blood. The addition of whole blood showed no detrimental effects to the lipid membrane.

10

EXAMPLE 4: Detection of endogenous ferritin in whole blood and serum

15

The bilayer biosensor is stable to whole blood addition and is fully functional for a single-step, endogenous ferritin detection in unprocessed blood. (Blood was obtained from a volunteer using CP2D as anticoagulant, which contains citric acid, sodium citrate, sodium acid phosphate, and dextrose).

20

1st layer: 9.3nM Gayy (Linker B)
5.5nM MSLXXB (MSL-C)
1.1uM MSLOH (MSL-D)
37uM MAAD
75uM DLP (Linker A)

25

2nd layer: 14mM (DPEPC:GDPE=7:3) : Ga5XB (Linker E) = 100,000:1

30

Electrodes were prepared and 2nd layer added as described in Example 3. Ferritin in either PBS, serum or whole blood was added to different electrodes [figures 10a), b), and c)], and the change of impedance with ferritin concentration was recorded. Figures 4 also shows the steps preceding the addition of analyte, i.e. when SA is added (5ul 1mg/ml in PBS), washing out excess SA, then in Figs 10 a), b), and c) only, the addition of anti-ferritin Fab' biotinylated at the thiol group (5ul 0.05mg/ml in PBS) and the subsequent rinsing step, is also shown.

35

Figs 10 d), e), and f) shows that in the absence of specific receptors for ferritin, there is no response, regardless of whether PBS, serum or whole blood was added. Note that no other reagents or washing steps are required after the sample containing the analyte has been added. The biosensor is manufactured up to the stage where specific receptors are added, then it is ready for the one-step addition of the sample.

EXAMPLE 5: Titration curve from endogenous ferritin in human serum

After clotting, blood obtained from 4 different volunteers was centrifuged down and serum was separated and used for the present example.

1st layer: 9.3nM Gayy (Linker B)
 5.5nM MSLXXXXB (MSL-C)
 1.1uM MSLOH (MSL-D)
 37uM MAAD
 75uM DLP (Linker A)
 2nd layer: 14mM (DPEPC:GDPE=7:3) : Ga5XB (Linker E) = 66,667:1

Electrodes were prepared and 2nd layer added as described in Example 4. From the initial well volume of 150ul, 100ul buffer was removed and replaced with 150ul serum. Different sera were added to different electrodes and the tau (s) of the ferritin response was calculated from the admittance at minimum phase. Figure 11 shows the response of the biosensor to sera containing one of the following concentrations of ferritin (measured using the Immulite autoanalyser); 18.8, 38, 280, or 476 pM, in duplicate.

Figs 10 d), e), and f) shows that in the absence of specific receptors for ferritin, there is no response, regardless of whether PBS, serum or whole blood was added. Note that no other reagents or washing steps are required after the sample containing the analyte has been added. The biosensor is manufactured up to the stage where specific receptors are added, then it is ready for the one-step addition of the sample.

EXAMPLE 6: Variation of analyte response with Gramicidin - biotin linker length and type

The linker length between gramicidin and biotin can be varied by adding varying numbers of caproyl groups, or by adding “multi-armed” linkers to gramicidin. each linker terminating with a biotin molecule, thus enabling a single gramicidin to capture either two biotin sites within the same SA molecule, or two or more SA molecules, depending on the length of the linker and number of “arms” present.

1st layer: 0.1uM Gayy (Linker B)
 10uM MSLXXB (MSL-C)
 0.8mM MAAD
 1mM DLP (Linker A)
 10mM GMPE
 2nd layer: Ga...B = 100,000:1, using either GaXB, GaXXB, GaXXXB, or Ga(XXX)₂ (Linkers E) 28mM GMPE

(Note: GMPE = GDPE with a monophytanyl chain instead of diphytanyl chains)

Electrodes were prepared and 2nd layer added as described in Example 4. SA was titrated into membranes containing the different types of gramicidin. Figure 12 shows the dependence of the SA response on linker type and length. The response was measured by normalising the frequency at the phase minima, i.e. calculating

$$(\text{phase}_{\text{final}} - \text{phase}_{\text{initial}}) / \text{phase}_{\text{final}}$$

EXAMPLE (7): Preparation of bilayer membrane

The structure of “linker lipid A” is shown in figure (1); the structure of “linker gramicidin B” is shown in figure (2); the structure of “membrane spanning lipid D” is shown in figure (3); the structure of “membrane spanning lipid C” where n=2 is shown in figure (3), the structure of “biotinylated gramicidin F” used is shown in figure (5), the structure of “biotinylated gramicidin E” used, where n=5, is shown in figure (4).

A glass slide or plastic support is evaporatively coated with a 50 angstrom chromium adhesion layer, followed by a 2000 angstrom layer of gold. The gold coated substrate is placed in an ethanolic solution containing "linker lipid A" (300 ul of 10 mM solution in ethanol), the disulfide of mercaptoacetic acid (150 ul of a 10 mM solution in ethanol), "linker gramicidin B" (100 ul of a 0.01 mg/ml solution in ethanol), "membrane spanning lipid D" (225 ul of a 1 mM solution in ethanol), "membrane spanning lipid C" (22.3 ul of a 0.01 mM solution in ethanol) and ethanol (50 ml). The gold coated substrate should preferably be placed into this solution within five minutes of preparation. The gold coated substrate is left in this solution for 60 minutes, and then rinsed with ethanol. The slide may then be stored in ethanol, water + sodium azide (0.01% w/v), ethylene glycol, glycerol, decane, decanol or hexadecane until required. When needed, the gold coated slide is rinsed with ethanol and is then assembled in an electrode holder such that an electrode is defined, that for the current examples has an area of approximately 16 mm². Then 5ul of a solution of 1,2-di(3RS,7R,11R-phytanyl)-glycero-3-phosphocholine and 1,2-di(3RS,7R,11R-phytanyl)glycerol in a 7:3 ratio, 14 mM total lipid concentration in ethanol is added to the surface of the gold electrode and then rinsed with two washes of 500 ul of phosphate buffered saline (PBS), leaving 100 ul PBS above the electrode surface. A counter electrode, typically silver, is immersed in the PBS solution; and the counter electrode and the sensing electrode are connected to an impedance bridge. A DC offset of -300 mV is applied to the sensing electrode during the AC measurement.

EXAMPLE (8). Preparation of solubilised gramicidin

Example (8A)

A solution of "linker gramicidin E" (1uM) and sodium dodecylsulfate (10uM) in PBS is sonicated in a bath sonicator for 20 minutes. This solution may be stored for at least 12 months at 4°C. Although the gramicidin with sodium dodecylsulfate is stable in aqueous solution, the gramicidin incorporates readily into sensing membranes and produces conducting ion channels. This change in conduction can be monitored using impedance spectroscopy.

Example (8B)

Alternatively, a solution of "linker gramicidin F" (20ul of 10uM in ethanol) was added to a solution of streptavidin (200ul of 1 mg/ml in PBS + 700 ul of PBS, total volume 1 ml) and mixed by vortexing for 1 minute. This solution is stable for several months at 4° C.

In the absence of either SDS or streptavidin, the ability for the gramicidin to insert into the bilayer membrane is deteriorates rapidly over one to two days. Not wishing to be bound by scientific theory, it is assumed that the gramicidin precipitates out of the aqueous solution.

EXAMPLE (9) Preparation of a biosensor membrane using solubilised gramicidin.Example (9A)

To the bilayer membrane prepared in example (7) is added a solution (10 ul) of solubilised gramicidin prepared in example 8A. The conductance of the membrane is monitored by impedance spectroscopy. This conductance increases as gramicidin molecules insert into the bilayer membrane. Addition of equivalent amounts of SDS or streptavidin without any gramicidin do not cause significant increases in conduction of the membrane. Prior to addition of the solubilised gramicidin the impedance at 10 Hz was 170 kohm/16mm². After addition of the solubilised gramicidin the impedance was monitored until the desired level of conductance had been achieved, (in this case an impedance of 41 kohms/16mm² at 10 Hz) the electrode well was rinsed with PBS (3x500ul). Streptavidin (5 ul of 0.1mg/ml in PBS) is added to the electrode well, left for three to five minutes and rinsed with PBS (3x 500 ul). In the case of a ferritin responsive sensor, biotinylated anti-ferritin Fab' (5ul of 0.06mg/ml in PBS) was added and after three to five minutes the electrode well was rinsed with PBS. In the case of a thyroid stimulating hormone (TSH) sensor a 1:1 mixture of two complementary biotinylated anti-TSH Fab's (10ul of 0.01 mg/ml) was added. The biotinylated Fab's were biotinylated via the free thiol group of freshly cleaved (Fab)₂ dimers. The sensor is now ready for addition of the analyte solution. Addition of a test solution of ferritin in PBS such that the final well concentration was 200 pM of ferritin gave an increase in impedance from 41

kohms/16mm² to 74 kohms/16mm². The impedance spectra are shown in figure 14.

Example (9B)

5 To the bilayer membrane prepared in example (7) is added a solution of streptavidin (5 ul of 0.1mg/ml in PBS). After three to five minutes the electrode well is rinsed with PBS and one of a complementary pair of biotinylated anti-TSH Fab' (10ul of 0.01mg/ml in PBS) is added. After 3 to 5 minutes the electrode is rinsed with PBS and a solution of solubilised
10 gramicidin as prepared in example (8B) (10ul) is added. The impedance of the membrane is monitored until the desired conduction is achieved (in this case an impedance of 49 kohm/16mm² at 10 Hz) and the electrode is then rinsed with PBS. The other of the complementary pair of biotinylated anti-TSH Fab' (10ul of 0.01mg/ml in PBS) is then added and after three to five
15 minutes the electrode is rinsed. The sensor now has the first of the complementary anti-TSH Fab' attached to the "membrane spanning lipid C" and the second of the complementary anti-TSH Fab' attached to "linker gramicidin F". Addition of a test solution of TSH in PBS such that the final analyte concentration in the well was 500 pM gave an increase of impedance
20 from 49 kohm/16 mm² to 60 kohm/16 mm². The impedance spectra are shown in figure 15.

EXAMPLE (10) Dry storage of sensor membrane

Example (10A)

Sensor membranes were prepared as in example (7).

The sensor membranes were then rinsed with a 0.5% (w/v) glycerol in water solution (+ 0.1% sodium azide). Excess glycerol solution was removed such that 20 ul of the glycerol solution was left in the electrode
30 well assembly. The sensor membrane was then placed in a chamber containing dessicant (RH in chamber was approximately 15%) and allowed to dry. The dry sensor membrane could then be stored at 15% -70% RH at room temperature for up to 1 week. It was found that when the membranes were dried from solutions with glycerol concentrations of less than 0.1% w/v
35 the membranes became excessively leaky on rehydration with PBS. Thus, the impedance at 10Hz for a freshly prepared, sealed membrane that has not

been dried was between 129-149 kohm/16mm², while the impedance at 10Hz for membranes that had been dried from 5-0.1% w/v glycerol solution was between 100-110 kohm/16mm². Membranes that had been dried from less than 0.1% w/v glycerol solution became very leaky with 10Hz impedances of less than 45 kohm.

Impedance at 10Hz (average of 16 electrodes):

| | Freshly prepared | 5 days at 15% RH |
|----------------------|------------------|------------------|
| 5% glycerol w/v | 148 kohm | 118 kohm |
| 10 0.5% glycerol w/v | 139 kohm | 98 kohm |
| 0.1% glycerol w/v | 148 kohm | 103 kohm |
| 0.05% glycerol w/v | 136 kohm | 35 kohm |

One of the advantageous properties of excess drying agent is that it protects the bilayer lipid membrane from passing through an air/water interface. The air/water interface may destabilise and disrupt certain lipid bilayer structures. The glycerol coating allows for controlled rehydration of the lipid membrane without the lipid bilayer immediately contacting the air/water interface as the analyte solution is added, as the rate of dissolution of the glycerol is slower than the rate of addition of the analyte solution.

It was found that if the sensor membrane was stored at <50%RH then on addition of analyte solution an equilibration/rehydration period occurred that lasted 30-90 seconds. This equilibration is not necessarily a problem when determining analyte concentration as it may be subtracted using a second non-sensing differential electrode. However, it is also possible to remove this equilibration/rehydration effect by pre-equilibrating the dry sensor membranes for a period of time in an atmosphere of RH of approximately 70%. Typically this pre-equilibration may be for 5-90 minutes prior to addition of analyte solution.

Example (10B)

Sensor membranes were prepared as in example (7) except that "linker gramicidin E" was incorporated into the bilayer at lipid:gramicidin ratio of 4,0000:1. The membranes were dried from 0.5%w/v glycerol solution as described in example (10A) and were subsequently stored for 5 days at room temperature at approximately 15%RH. The sensor membranes were

then rehydrated with PBS solution and streptavidin was added (5ul of 0.1mg/ml). The rate of increase in the impedance was measured. A convenient measure was the frequency at the minimum phase taken from a standard Bode plot of the phase versus time. An exponential curve ($y = -ke^{t/\tau}$) was fitted to the response rate curve and as a measure of the rate of gating towards the streptavidin the tau value was used. The tau value is related to the analyte concentration. It was found that over a period of five days the tau value in response to streptavidin gating did not vary within experimental error. Thus:

10

| <u>Days of Storage</u> | <u>Tau (s), (std. deviation)</u> |
|------------------------|----------------------------------|
| 0 | 71 (26) |
| 1 | 79 (14) |
| 2 | 56 (12) |
| 15 | 3 |
| 5 | 80 (27) |
| | 77 (16) |

20

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

CLAIMS:

1. A method of producing a first layer electrode membrane comprising:-
 - (1) Forming a solution containing Linker Lipid A, the disulfide of mercaptoacetic acid (MAAD) or similar molecule, linker Gramicidin B, membrane spanning lipid C (MSL-C) and membrane spanning lipid D (MSL-D) or other suitable linker molecules and other ion channel or ionophore combinations;
 - (2) Contacting an electrode containing a clean gold surface with the solution, the disulfide containing components in the solution thus adsorbing onto the gold surface of the electrode;
 - (3) Rinsing the electrode with a suitable organic solvent; and
 - (4) Removing the excess organic solvent used for rinsing.
2. A method according to claim 1, wherein the solution contains the disulfide of mercaptoacetic acid (MAAD) or 2-mercaptoethanol (EDS).
3. A method according to claim 2, wherein the ratio of Linker Lipid A to the disulfide of mercaptoacetic acid (MAAD) or 2-mercaptoethanol (EDS) is 2:1.
4. A method according to claim 2 or 3, wherein the ratio of (Linker Lipid A + MAAD or EDS) to MSL-D is in the range of 10:1 to 100:1.
5. A method according to any one of claims 2 to 4, wherein the ratio of (Linker Lipid A + MAAD or EDS) to MSL-C is between 20,000:1 and 100:1.
6. A method according to any one of claims 2 to 5, wherein the ratio of (Linker Lipid A + MAAD or EDS) to MSL-C is 20,000:1.
7. A method according to any one of claims 2 to 6, wherein the solution contains linker Gramicidin B rather than another suitable linker molecule/ion channel or other combination.
8. A method according to claim 7, wherein the ratio of (Linker Lipid A + MAAD or EDS) to linker Gramicidin B is 10,000:1.

9. A method according to claim 7 or 8. wherein the ratio of (Linker Lipid A + MAAD or EDS) to linker Gramicidin B is between 20.000:1 and 100,000:1.

5 10. A method according to any one of the preceding claims wherein the gold electrode consists of a freshly evaporated or sputtered gold electrode.

10 11. A method according to claim 10. wherein the gold electrode surface is freshly cleaned using a plasma etching process or an ion beam milling process.

15 12. A method according to any one of the preceding claims wherein the solvent for the adsorbing solution (step (1)) and for the rinsing step (4) is ethanol.

13. A method of producing a monolayer electrode membrane comprising:-

20 (1) Forming a solution containing the disulfide of mercaptoacetic acid (MAAD) or similar molecule, membrane spanning lipid C (MSL-C) and/or membrane spanning lipid D (MSL-D) and, optionally, Linker Lipid A, linker Gramicidin B or other suitable linker molecules and other ion channel combinations;

25 (2) Contacting an electrode containing a clean gold surface with the solution, the disulfide containing components in the solution thus adsorbing onto the gold surface of the electrode;

(3) Rinsing the electrode with a suitable organic solvent; and

(4) Removing the excess organic solvent used for rinsing, wherein the solution in step (1) contains more than a molar % of 50% of a membrane spanning lipid.

30 14. A method according to claim 13. wherein the solution in step (1) contains more than a molar % of 70% of a membrane spanning lipid, 29% MAAD or 2-mercaptoethanol (EDS) and 1% other membrane spanning lipids.

35 15. A method according to claim 13 or 14, wherein the gold electrode consists of a freshly evaporated or sputtered gold electrode.

16. A method according to claim 15 wherein the gold electrode surface is freshly cleaned using a plasma etching process or an ion beam milling process.

5

17. A method according to any one of claims 13 to 16, wherein the solvent for the adsorbing solution (step (1)) and for the rinsing step (4) is ethanol.

10

18. A method according to any one of claims 13 to 17, wherein MAAD, or similar spacer molecule, such as EDS is covalently linked to the membrane spanning lipids C or D.

15

19. A method according to any one of claims 13 to 18, wherein MSL-C or D is covalently linked to DPEPC, GDPE, triphytanyl or similar molecule.

20

20. A method according to any one of claims 13 to 19, wherein the solution contains the disulfide of mercaptoacetic acid (MAAD) or 2-mercaptoethanol (EDS).

21. A method according to claim 24, wherein MSL-C or D is covalently linked to MAAD or EDS and DPEPC, GDPE, triphytanyl or similar molecule.

25

22. A method of producing a second layer electrode membrane combination comprising:-

30

(1) Adding a solution of lipid and biotinylated gramicidin E dispersed in a suitable solvent onto the electrode surface containing a first layer produced by a method according to any one of claims 1 to 12;

(2) Rinsing the electrode surface with an aqueous solution;

(3) Adding a solution of streptavidin, avidin, neutravidin, avidin or streptavidin derivative;

(4) Rinsing the electrode with an aqueous solution in order to remove excess streptavidin, avidin, neutravidin, or other avidin or streptavidin derivative;

35

(5) Adding a solution of a biotinylated binding partner molecule; and

- (5) Adding a solution of a biotinylated binding partner molecule;
and
(6) Rinsing the coated electrode with an aqueous solution.

5 23. A method according to claim 22, wherein the lipid used in step (1) is a mixture of diphytanyl phosphatidyl choline (DPEPC) and glyceryl diphytanyl ether (GDPE).

10 24. A method according to claim 22 or 23, wherein the DPEPC and GDPE is in a 7:3 ratio.

25. A method according to claim 22, wherein the lipid used in step (1) is a triphytanyl phosphoryl choline as shown in Figure (6).

15 26. A method according to claim 22, wherein 0-50% cholesterol is incorporated into the lipids used in step (1).

20 27. A method according to claim 22, wherein 0-20% cholesterol is incorporated into the lipids used in step (1)

28. A method according to any one of claims 22 to 27, wherein the ratio of lipid to biotinylated gramicidin E is between 10,000:1 and 1,000,000:1.

25 29. A method according to claim 28, wherein the ratio of lipid to biotinylated gramicidin E is 100,000:1.

30 30. A method according to any one of claims 22 to 29, wherein the biotin is attached to the gramicidin via the ethanolamine end using a linker arm that is made up of between 1-8 aminocaproyl groups.

35 31. A method according to any one of claims 22 to 30, wherein two biotins are attached to the gramicidin at the ethanolamine end such that the biotins are able to bind simultaneously into the adjacent binding sites of a streptavidin, avidin or a similar biotin-binding protein.

32. A method according to any one of claims 22 to 30, wherein two biotins are attached to the gramicidin at the ethanolamine end such that the biotins are able to bind simultaneously into two separate streptavidin, avidin or a similar biotin-binding protein molecules.

5

33. A method according to claim 31, wherein the two biotins are attached to the gramicidin via the ethanolamine end such that each biotin is attached to 2 to 4 linearly joined aminocaproyl groups that are attached to a lysine groups as shown in Figure (5).

10

34. A method according to claim 31, wherein the biotins are attached to the gramicidin via the ethanolamine end such that each biotin is attached to 2 to 20 linearly joined aminocaproyl groups that are attached to a lysine groups.

15

35. A method according to claim 30, wherein the biotins are attached to the gramicidin via the ethanolamine end such that each biotin is attached to 2 to 20 aminocaproyl groups arranged as a branched structure.

20

36. A method according to any one of claims 22 to 33, wherein the amount of streptavidin, avidin or other similar biotin-binding protein that is added in step (3) is sufficient to cause a prozone effect, allowing most of the available biotinylated species in the membrane to have one streptavidin or related molecule bound to prevent cross-linking between gramicidin channels and MSL until a sample containing analyte is added to the sensor.

25

37. A method according to any one of claims 22 to 36, wherein prior to the addition of streptavidin, avidin or similar biotin-binding protein, the lipid membrane electrode assembly is cooled.

30

38. A method according to claim 37, wherein the lipid membrane electrode assembly is cooled to between 0° and 50°C.

35

39. A method according to claim 38, wherein the lipid membrane electrode assembly is cooled to between 0° and 5°C.

40. A method according to any one of claims 37 to 39, wherein the subsequent rinsing and addition of the biotinylated binding partner molecule is carried out at 0° to 50°C.
- 5 41. A method according to claim 40, wherein the subsequent rinsing and addition of the biotinylated binding partner molecule is carried out at 0° to 5°C.
- 10 42. A method according to claims 40 or 41, wherein the binding partner molecule is a biotinylated antibody or biotinylated antibody fragment.
43. A method according to any one of claims 37 to 39, wherein the binding partner molecule is a Fab' fragment that is biotinylated via the free Fab' thiol group.
- 15 44. A method according to claim 40, wherein the linker between the Fab' and biotins is between 10-80 angstroms in length.
- 20 45. A method according to claim 42 or 43, wherein the linker between the Fab' and biotins consists in 1-8 aminocaproyl groups.
- 25 46. A method according to claim any one of claims 42 to 45, wherein the group containing two biotins is attached to the antibody or antibody fragment such that the two biotins are able to simultaneously complex one streptavidin, avidin or other similar biotin-binding proteins.
- 30 47. A method according to claim any one of claims 42 to 45, wherein the group containing two biotins is attached to the antibody or antibody fragment such that the two biotins are able to complex simultaneously two streptavidin, avidin or other similar biotin-binding proteins.
48. A method according to any one of claims 22 to 41, wherein steps (3) to (5) are substituted with:
- 35 (3) Adding a solution containing a conjugate between streptavidin, avidin, neutravidin or other avidin or streptavidin derivative and a molecule which is a member of a binding pair.

49. A method according to claim 48 wherein the binding partner molecule is an antibody or an antibody fragment such as a Fab, Fab' or Fabv fragment.

5

50. A method according to claim 48 or 49, wherein the binding pairs are selected from: naturally occurring binding proteins and cellular receptors/analytes, enzymes or enzyme analogues/substrates, lectins/carbohydrates, complementary nucleic acid sequences. Anti-FC, Protein A or Protein G/antibody.

10

51. A method of producing a second layer electrode membrane combination comprising:-

15

(1) Adding a solution of lipid dispersed in a suitable solvent onto the electrode surface containing a first layer produced according to any one of claims 1 to 12;

(2) Rinsing the electrode surface with an aqueous solution;

(3) adding an aqueous solution containing ionophore co-dispersed with detergent or solubilised by coupling to a high molecular weight species;

20

(4) Rinsing the electrode with an aqueous solution; and

(5) Adding the receptor using either streptavidin, avidin or other similar biotin-binding protein followed by addition of a biotinylated antibody or antibody fragment or adding a streptavidin, avidin or other similar biotin-binding protein conjugated to an antibody or antibody fragment.

25

52. A method according to claim 51, wherein the lipid used in step (1) is a mixture of diphytanyl phosphatidyl choline (DPEPC) and glyceryl diphytanyl ether (GDPE).

30

53. A method according to claim 52. wherein the DPEPC and GDPE is in a 7:3 ratio.

54. A method according to claim 51, wherein the lipid used in step (1) is a triphytanyl phosphoryl choline as shown in Figure (6).

35

55. A method according to any one of claims 51 to 54, wherein the membrane produced also contains 0-50% cholesterol.

56. A method according to any one of claims 51 to 54, wherein the
5 membrane produced also contains 0-20% cholesterol.

57. A method according to any one of claims 51 to 56, wherein the
aqueous solution used in step (3) contains gramicidin or a gramicidin
derivative that is added to an aqueous solution of a detergent such that the
10 detergent is present in excess relative to the gramicidin but wherein the total
concentration of the detergent is below the critical micelle concentration
(CMC).

58. A method according to claim 57, wherein the gramicidin/detergent
15 solution is sonicated using an ultrasonic bath or horn for 5-20 minutes.

59. A method according to claim 57 or 58, wherein the detergent is
selected from sodium dodecylsulfate, octylglucoside, tween, and other ionic
or non-ionic detergents.
20

60. A method according to claim 59, wherein the detergent is sodium
dodecylsulfate.

61. A method according to claim 60, wherein the concentration of the
25 sodium dodecylsulfate is less than 0.00001M and the concentration of
gramicidin is 10 times less than the sodium dodecylsulfate concentration.

62. A first layer membrane electrode combination comprising an
electrode and a first layer membrane comprising a closely packed array of
30 amphiphilic molecules and a plurality of ionophores, the first layer
membrane being connected to the electrode by means of a linker group, said
first layer membrane being stored in the presence of a solvent.

63. An electrode combination according to claim 62, wherein the solvent
35 in which the electrodes are stored is an organic solvent or an aqueous
solvent.

5 64. An electrode combination according to claim 63, wherein the solvent in which the electrodes are stored is selected from ethanol, glycerol, ethylene glycol and alcohols or diols containing between 3 to 12 carbon atoms.

10 65. An electrode combination according to claim 63, wherein the solvent in which the electrodes are stored is a hydrocarbon with between 8 to 20 carbon atoms.

15 66. An electrode combination according to claim 63, wherein the solvent is an aqueous solution containing a detergent.

20 67. An electrode combination according to claim 62 or 63, wherein the solvent in which the electrodes are stored is a compound that is able to coat the electrodes such that oxidation of the electrode surface is minimised.

25 68. An electrode combination according to claim 67, wherein the solvent can be applied as a thin film.

30 69. A lipid membrane based biosensor comprising a lipid membrane incorporating ionophores, the conductivity of the membrane being dependent on the presence or absence of an analyte, wherein the aqueous bathing solution in which the biosensor normally resides, is removed in a manner such that on drying of said lipid membrane biosensor, the lipid membrane and the receptor molecules retain their function, structure and activity, when rehydrated.

35 70. A biosensor according to claim 69, such that during the drying process the biosensor membrane does not have contact with the air-water interface.

71. A biosensor according to claim 69, such that during the rehydration process the biosensor membrane does not have contact with the air-water interface.

72. A biosensor according to any one of claims 69 to 71, wherein the aqueous bathing solution is removed by a method of drying selected from lyophilisation, evaporation, and evaporation over controlled humidity.

5 73. A biosensor according to any one of claims 69 to 72, wherein the aqueous bathing solution is replaced with a water replacing substance selected from protein, low molecular weight diols or triols, polyethylene glycol, low molecular weight sugars, polymeric peptides, polyelectrolyte and combinations thereof.

10 74. A biosensor according to claim 73, wherein the water replacing substance is selected from bovine serum albumin, serum, fish gelatin, non-fat dry milk powder, casein, glycerol, ethylene glycol, diethylene glycol, polyethylene glycol, trehalose, xylose, glucose, sucrose, dextrose, dextran or
15 ficoll.

75. A biosensor according to claim 74, wherein the water replacing substance is selected from glycerol, sucrose, dextran or trehalose.

20 76. A biosensor according to claim 73, wherein the class of molecules are chosen such that they have the additional advantage of providing a spreading layer for the sample.

25 77. A biosensor according to claim 73, wherein the class of molecules are chosen such that they have the additional advantage of providing filter against specific cells, bacteria, viruses and classes of molecules, such as large molecular weight proteins.

30 78. A biosensor according to claim 73, wherein the class of molecules are chosen such that they have the additional advantage of providing a reservoir for specific displacement reagents, which are required to compete off small analytes bound to proteins in serum or blood.

35 79. A biosensor according to claim 73, where any of water replacing agents may be bound covalently to specific membrane components.

5 81. A method according to any one of claims 13 to 21, wherein valinomycin is covalently linked to the MSL-C or MSL-D via a linker of appropriate length to permit the valinomycin to diffuse from one side of the membrane to another.

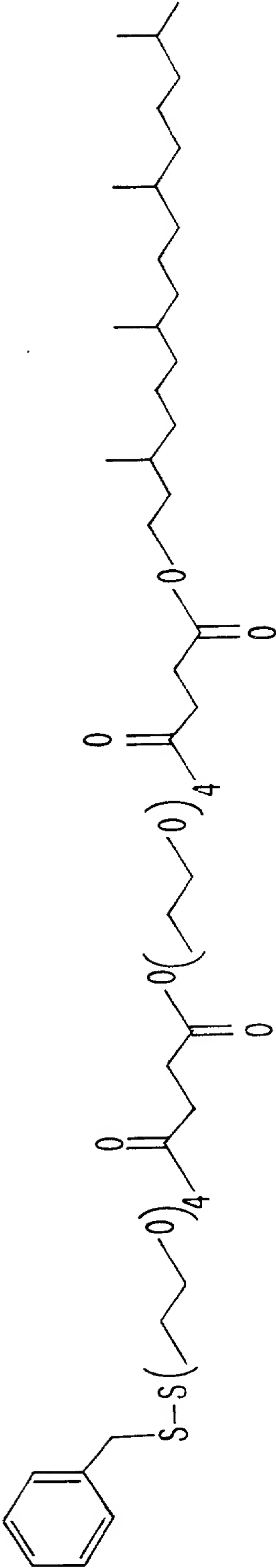
10 82. A method according to claim 1 or 2, wherein the ratio of Lipid Linker A to the disulfide of mercaptoacetic acid (MAAD) or 2-mercaptoethanol (EDS) is in the range of 5:1 to 1:2.

ABSTRACT

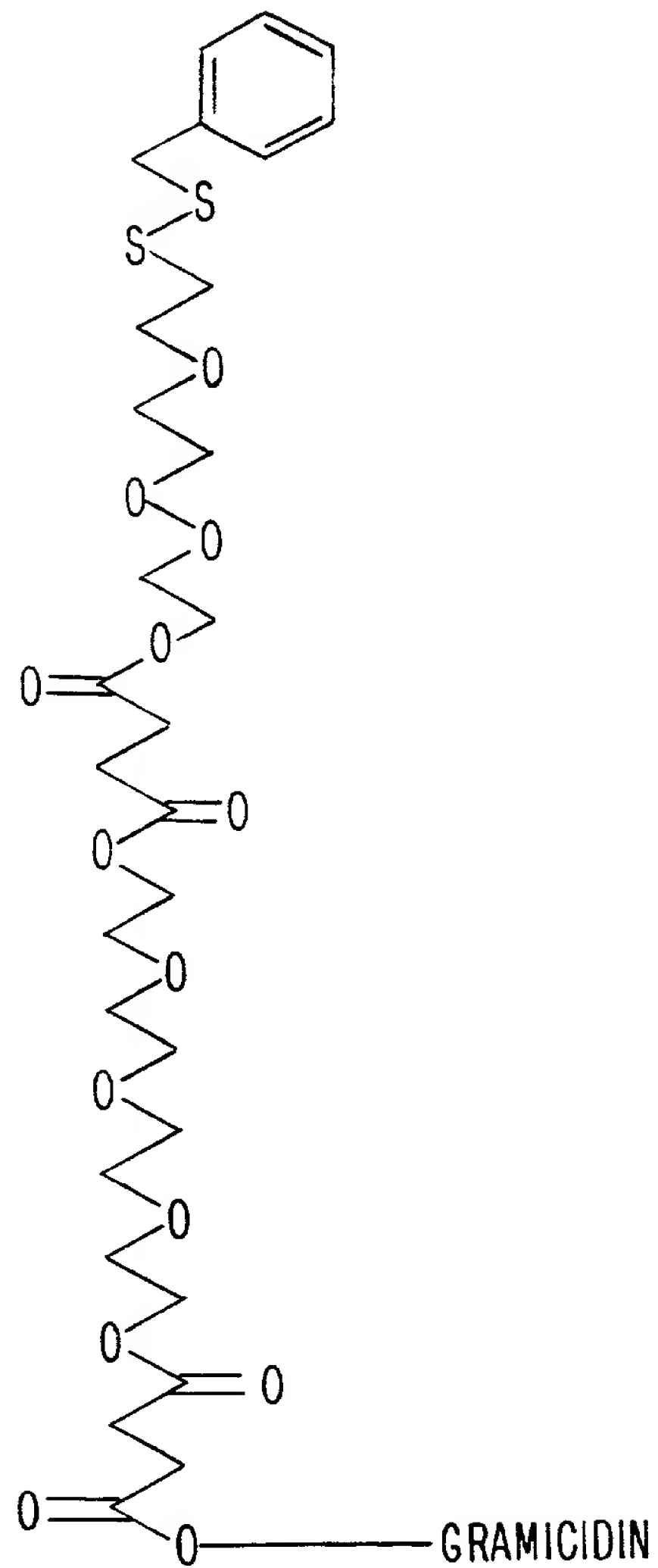
Electrode membrane combinations for use in biosensors to detect analytes in a sample and methods for making and storing same are disclosed. In one aspect, a method is provided for producing a first layer electrode membrane comprising:-

- (1) Forming a solution containing Linker Lipid A, the disulfide of mercaptoacetic acid (MAAD) or similar molecule, linker Gramicidin B, membrane spanning lipid C (MSL-C) and membrane spanning lipid D (MSL-D) or other suitable linker molecules and other ion channel combinations;
- (2) Contacting an electrode containing a clean gold surface with the solution, the disulfide containing components in the solution thus adsorbing onto the gold surface of the electrode;
- (3) Rinsing the electrode with a suitable organic solvent; and
- (4) Removing the excess organic solvent used for rinsing.

FIG. 1

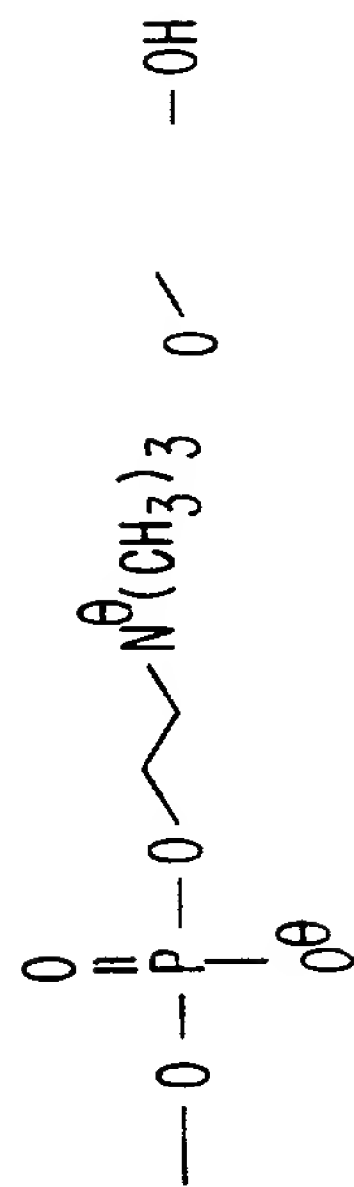
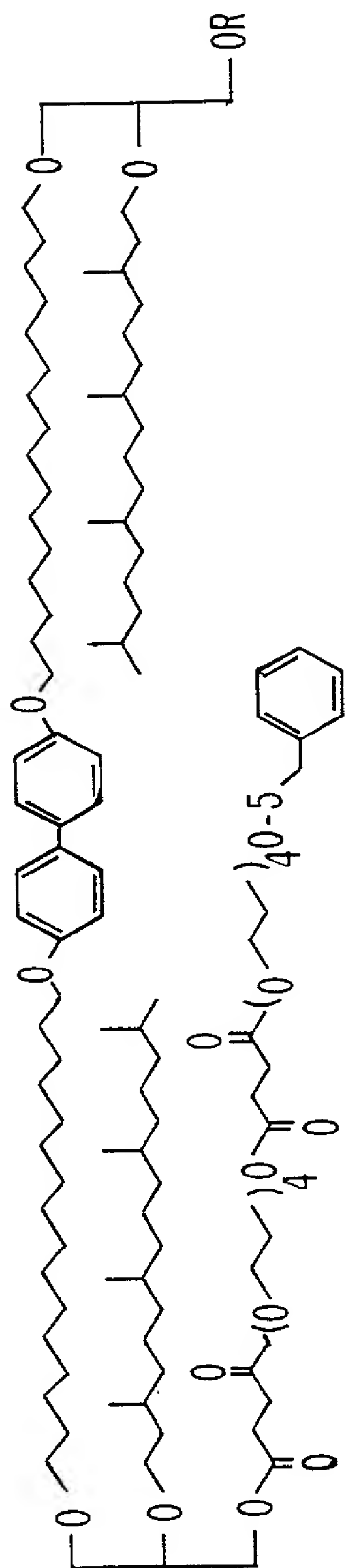


LINKER LIPID A

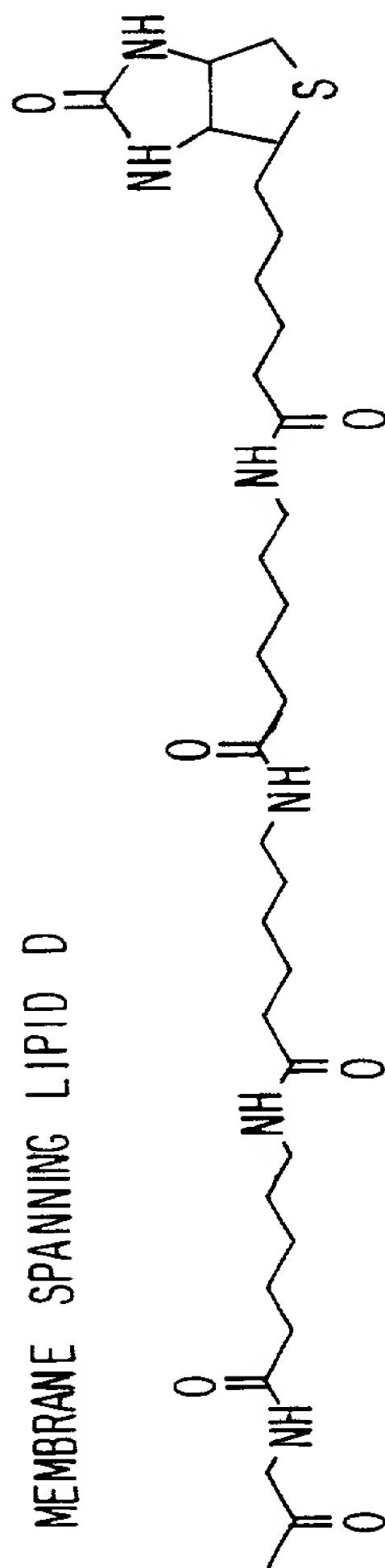


LINKER GRAMICIDIN B

FIG. 2



MEMBRANE SPANNING LIPID D



MEMBRANE SPANNING LIPID C

FIG. 3

FIG. 7A

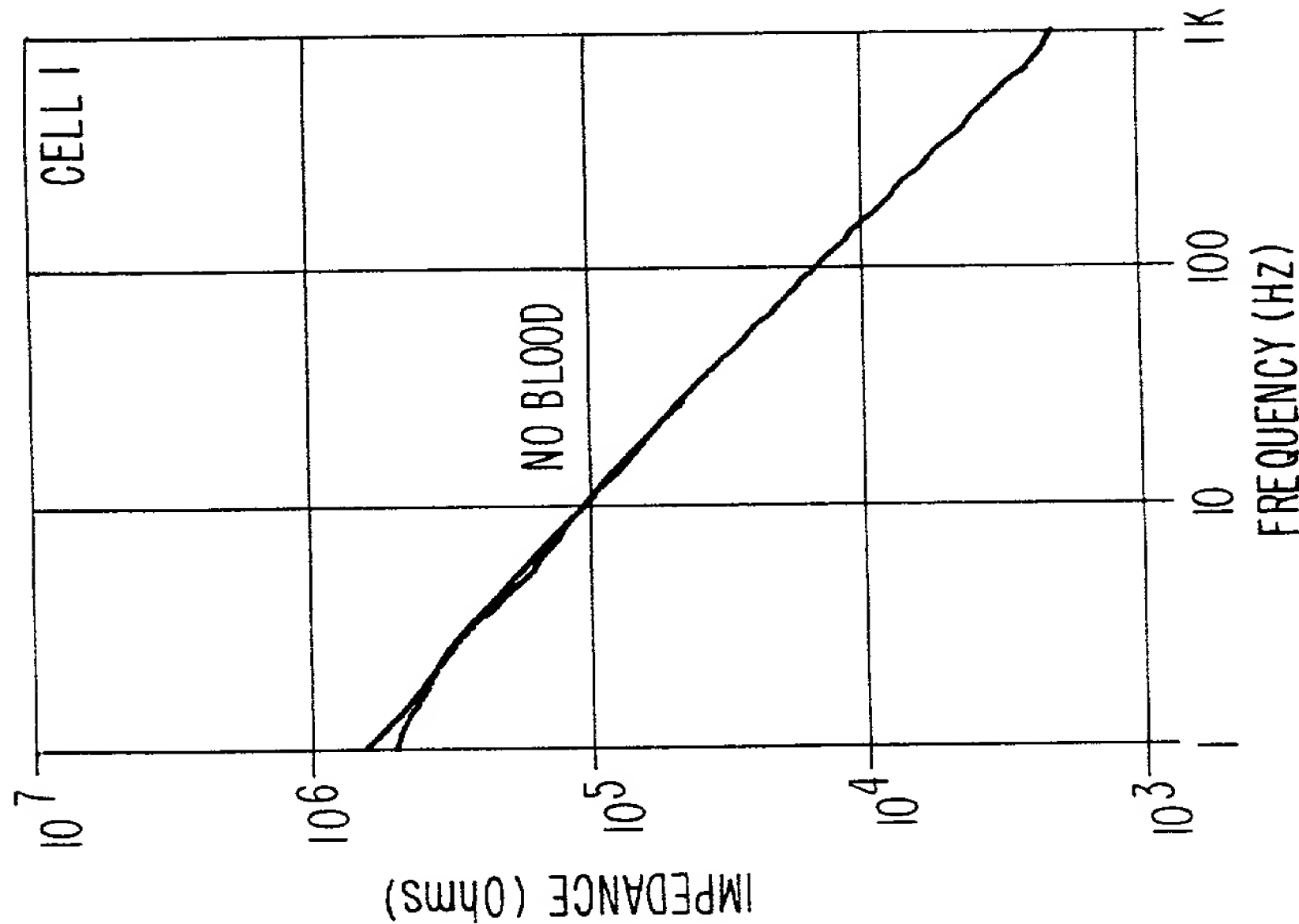


FIG. 7B

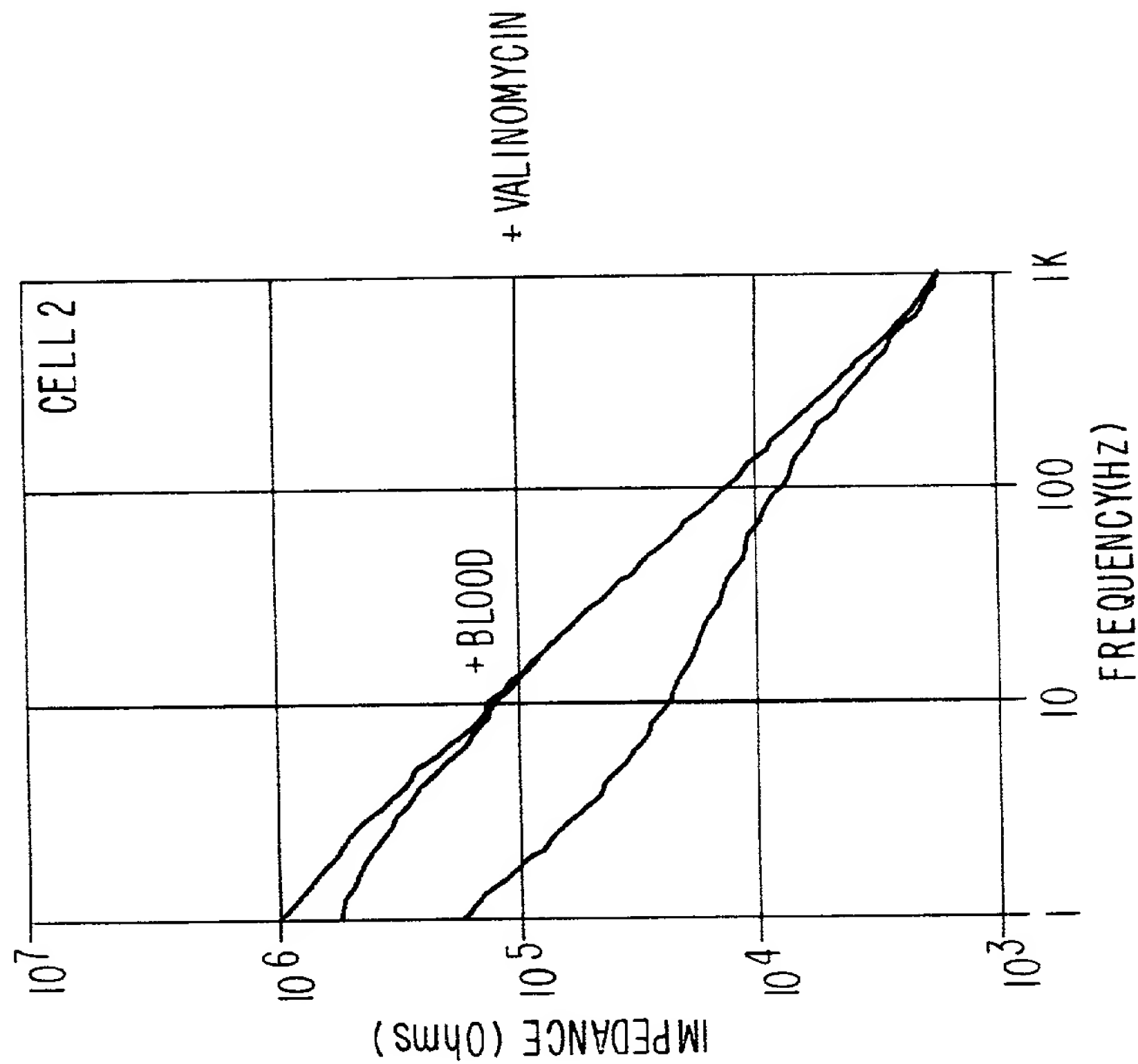


FIG. 7C

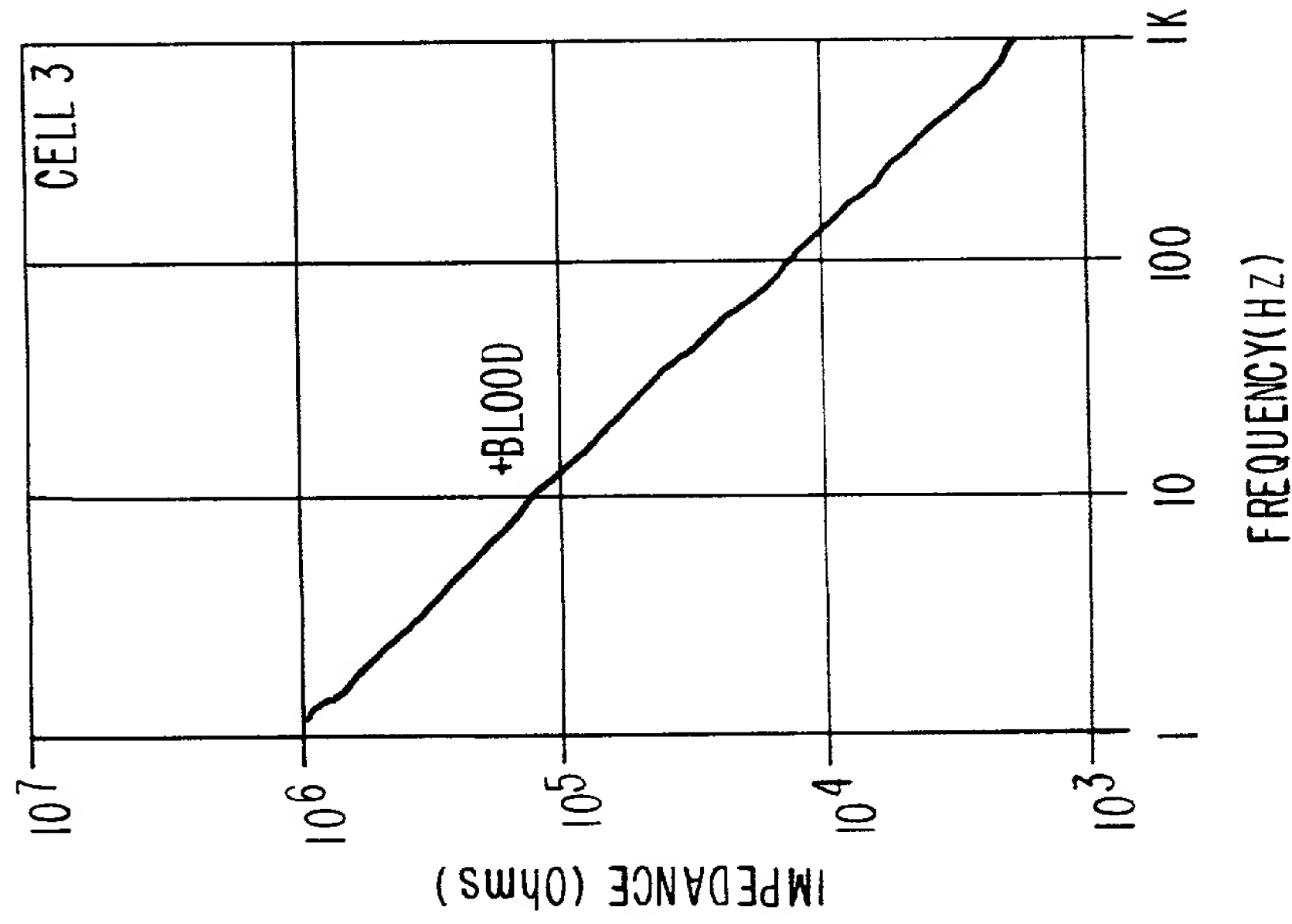
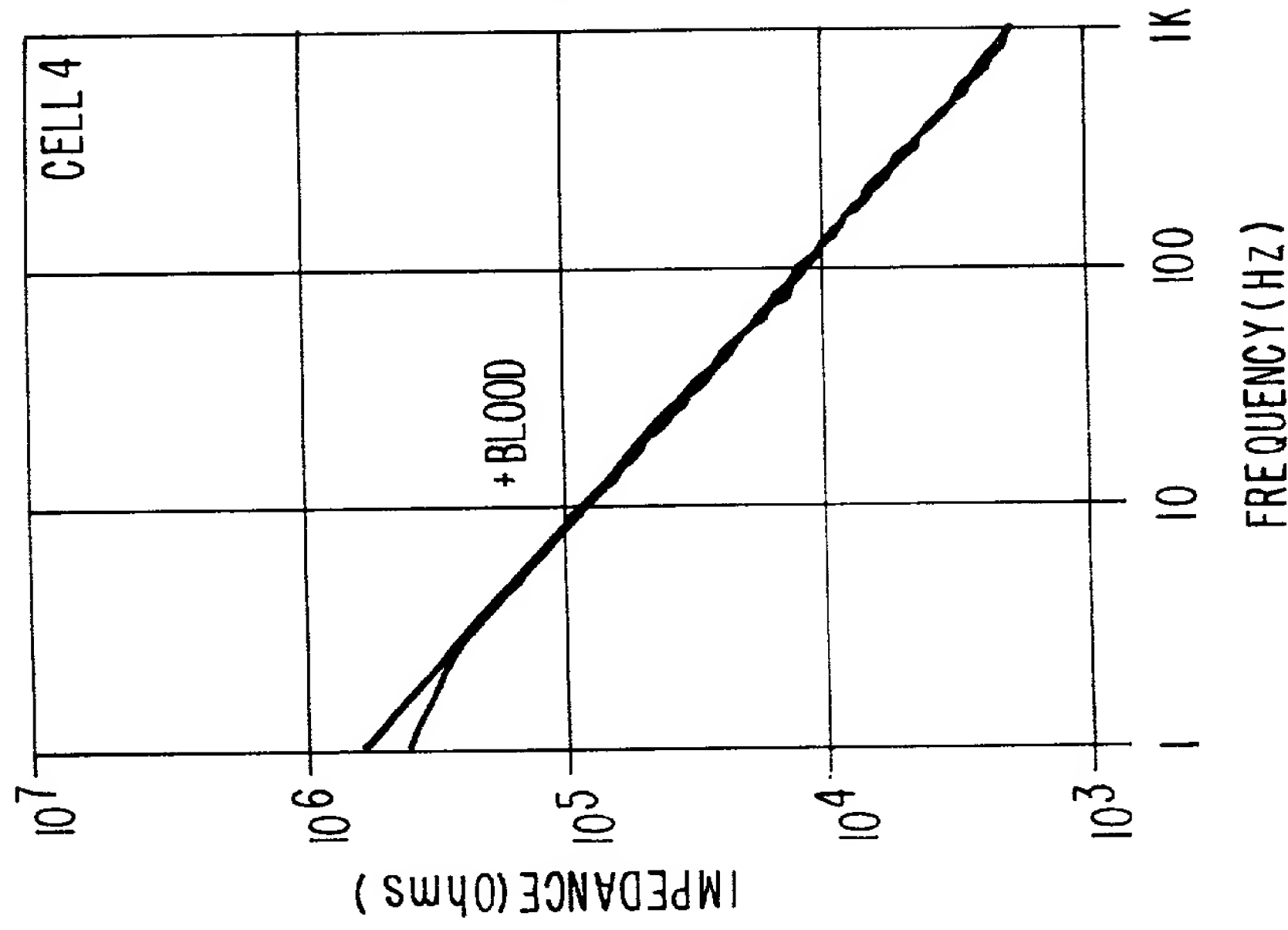


FIG. 7D



6/15
NO VALINOMYCIN

FIG. 8

REPEATED CHALLENGE WITH BLOOD:
ALL MONOLAYERS, PBS WASH, + mM VAL

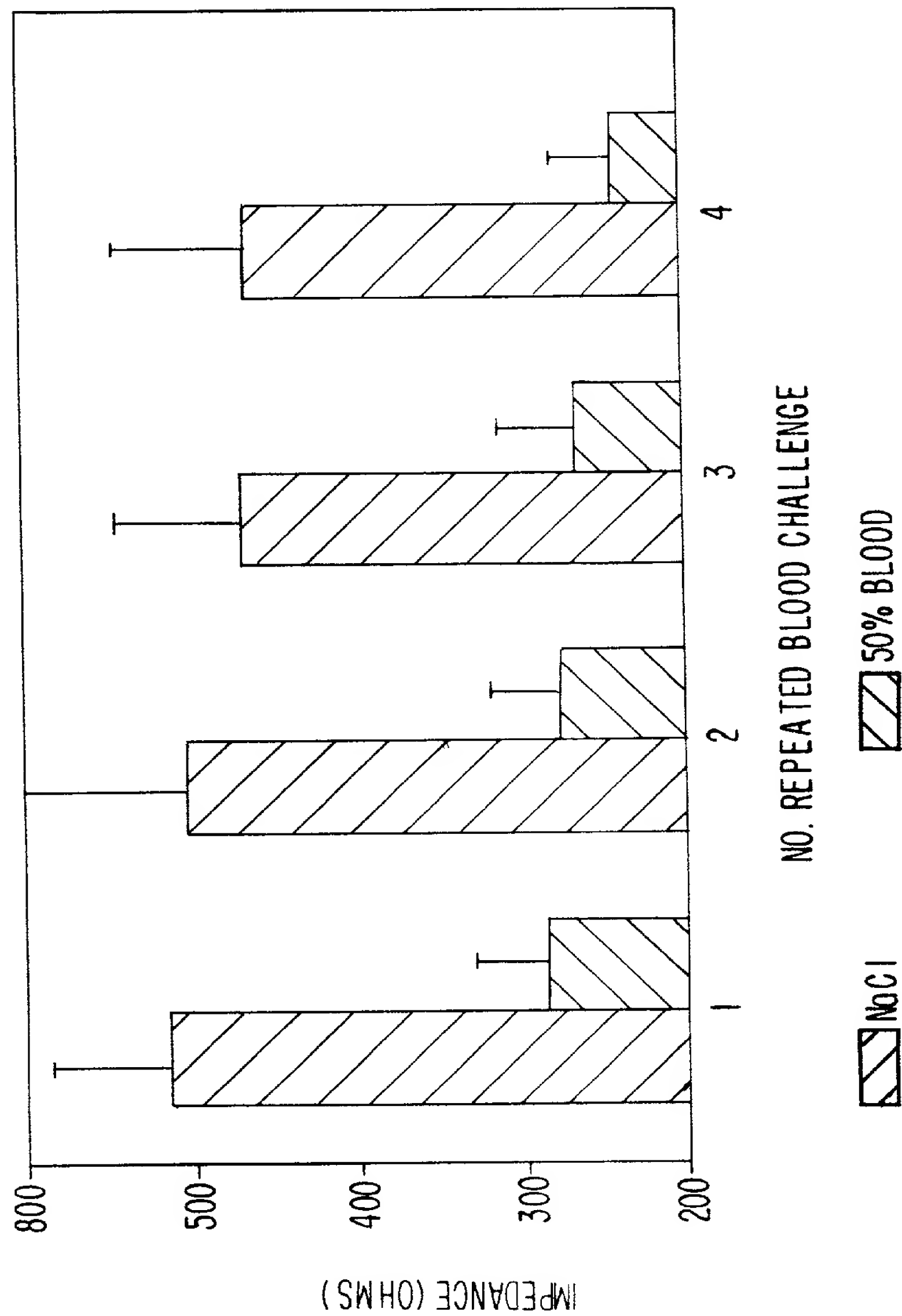
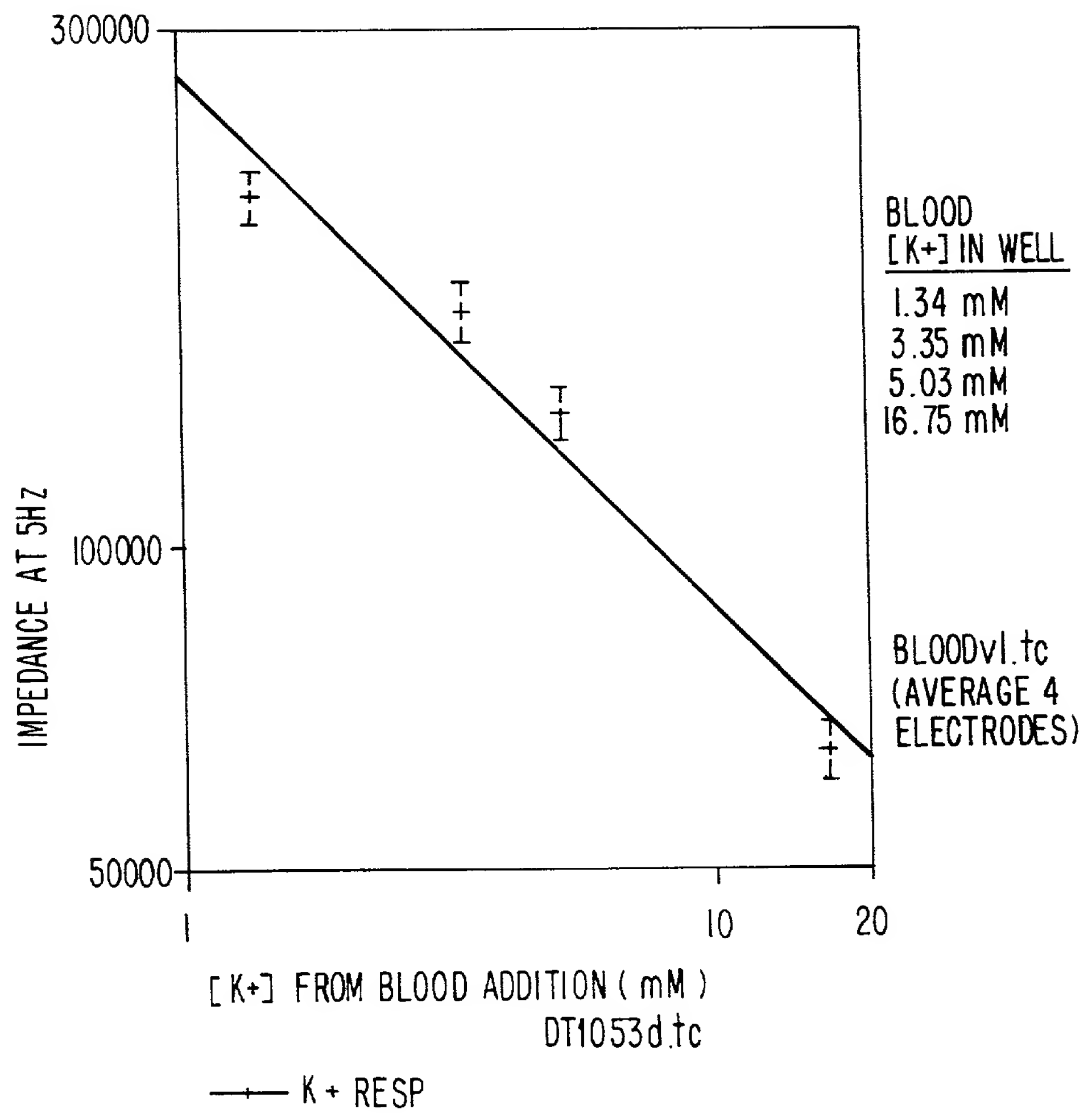
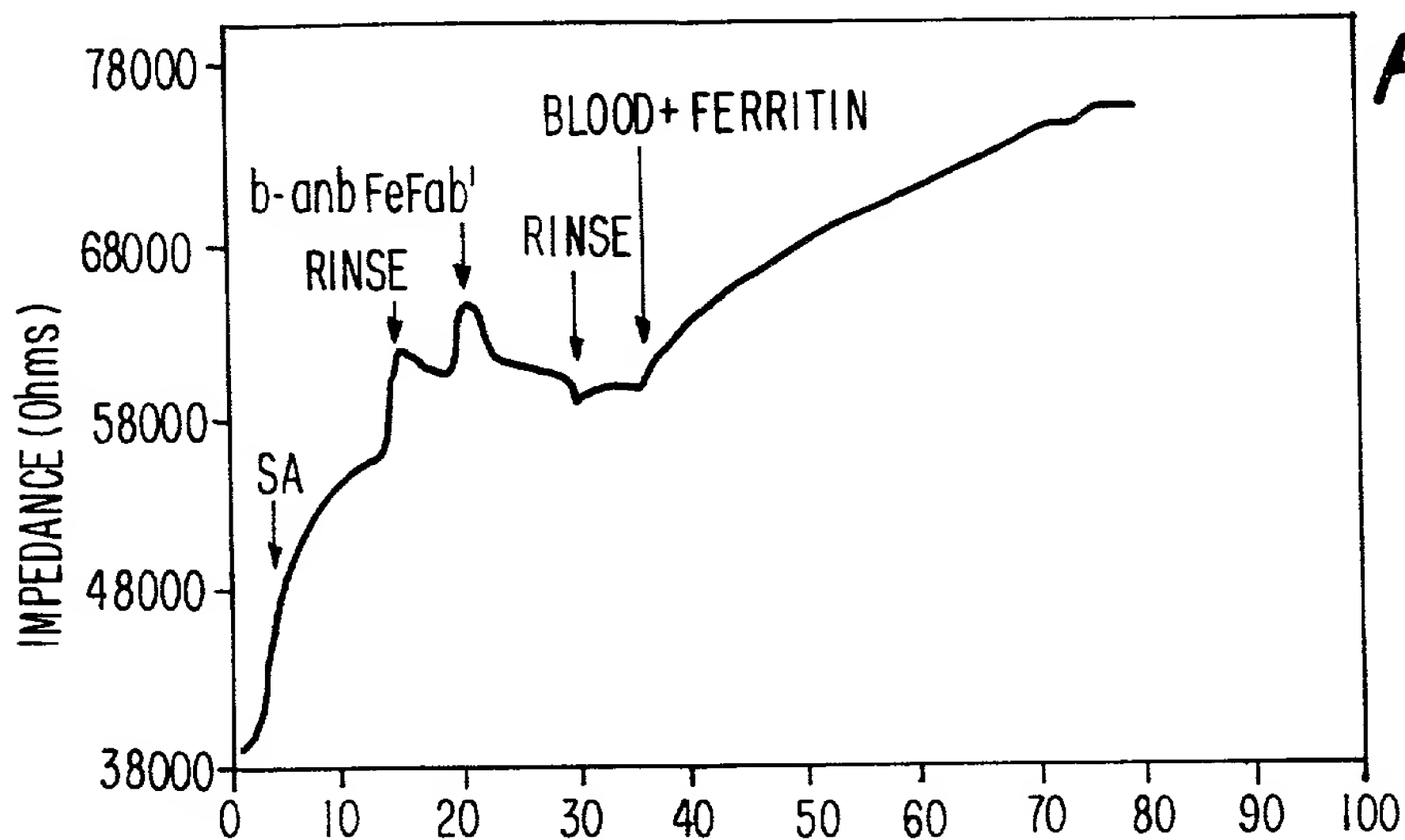
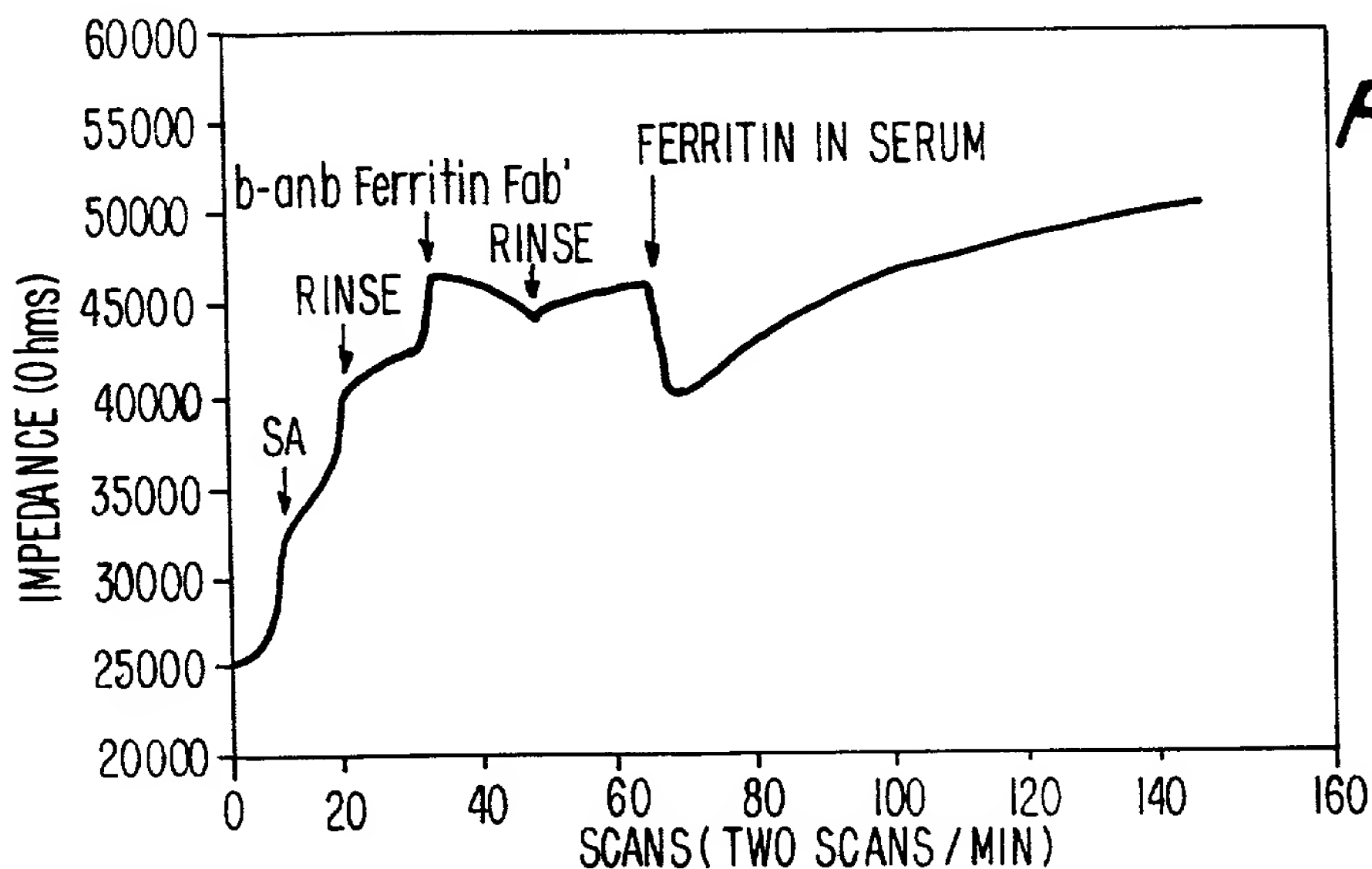
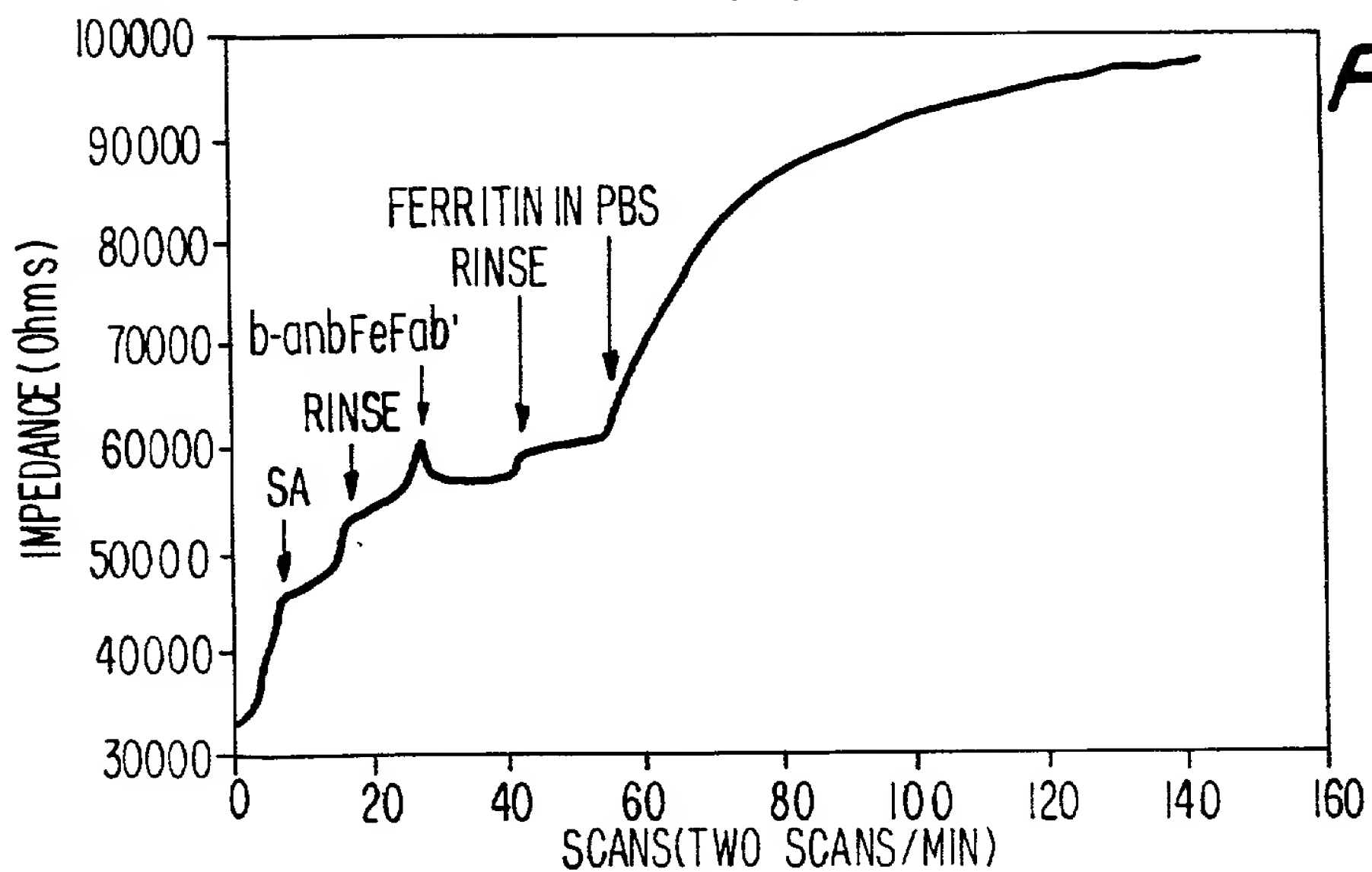


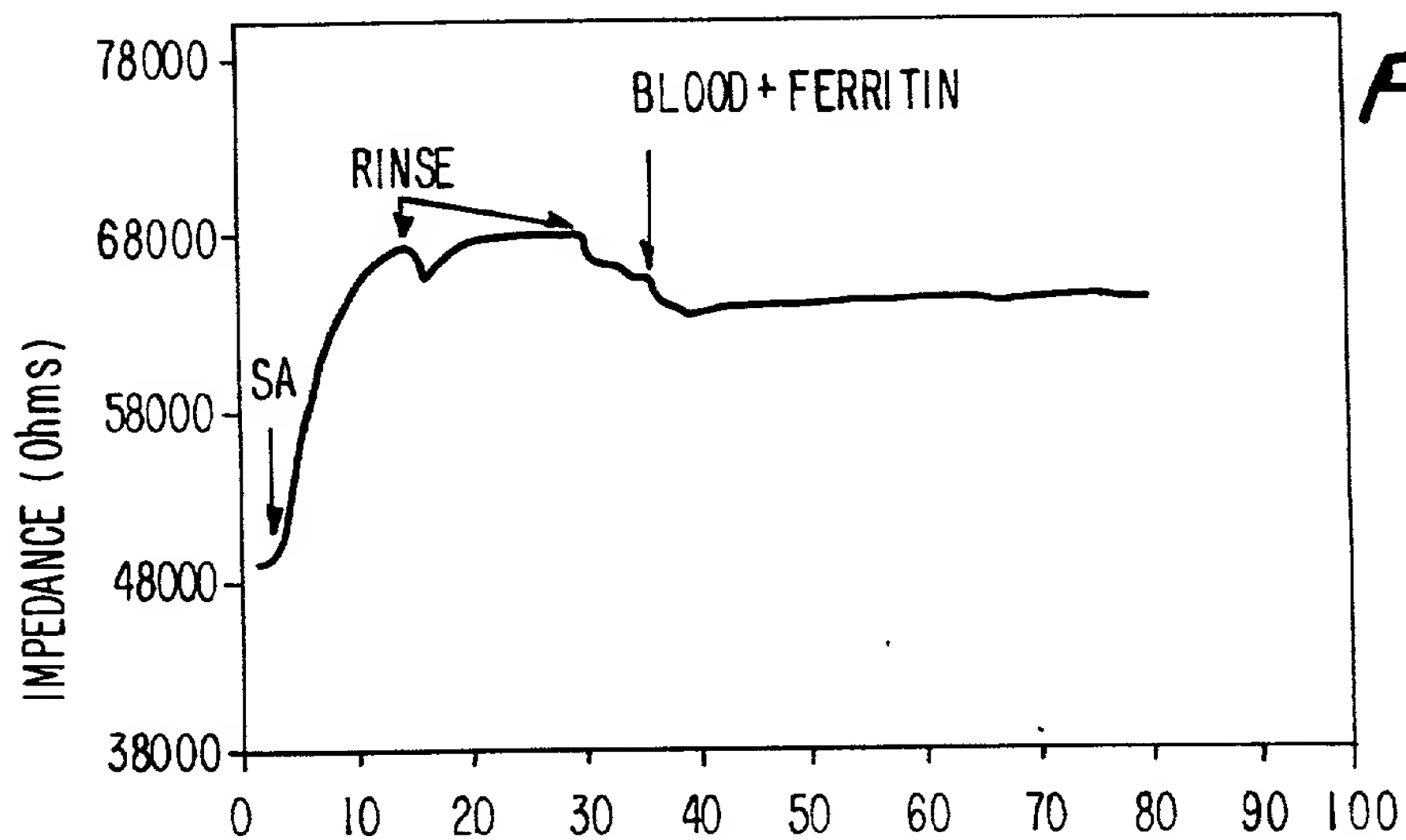
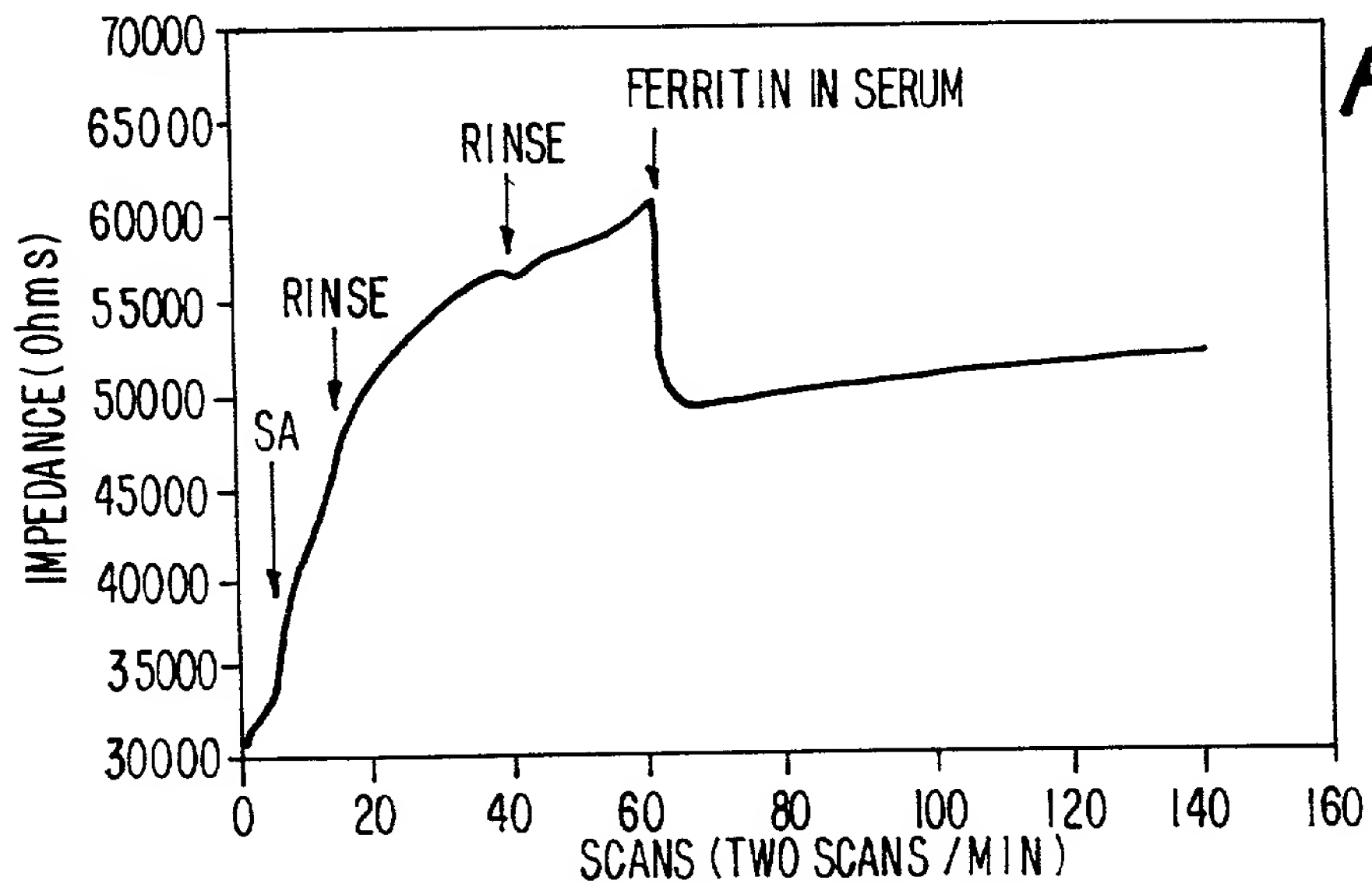
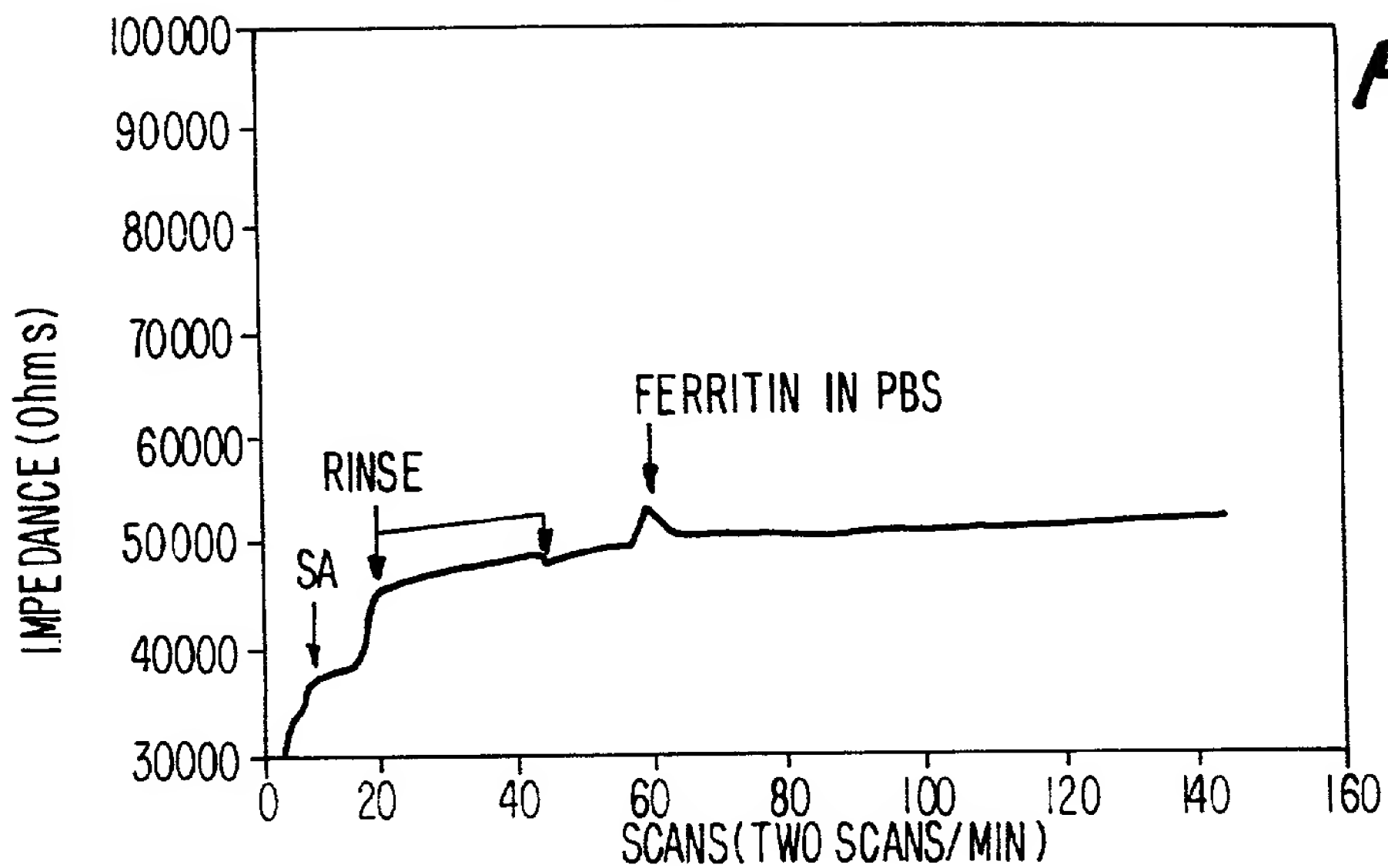
FIG. 9

BLOOD-VOLUME TITRATION



9/15





11/15

FIG. 11

ENDOGENOUS SERUM FERRITIN DETECTION
FROM 4 DIFFERENT VOLUNTEERS

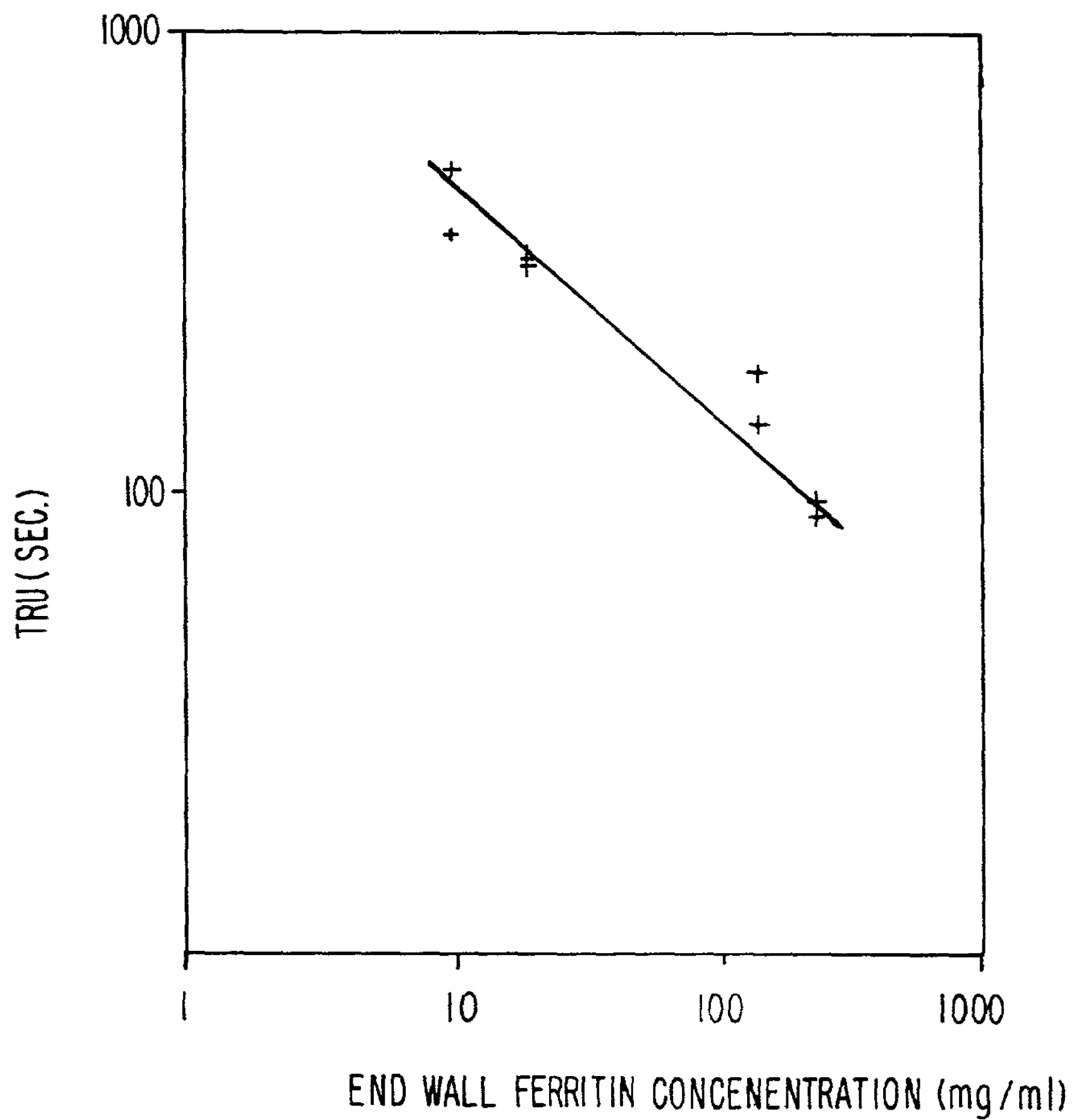
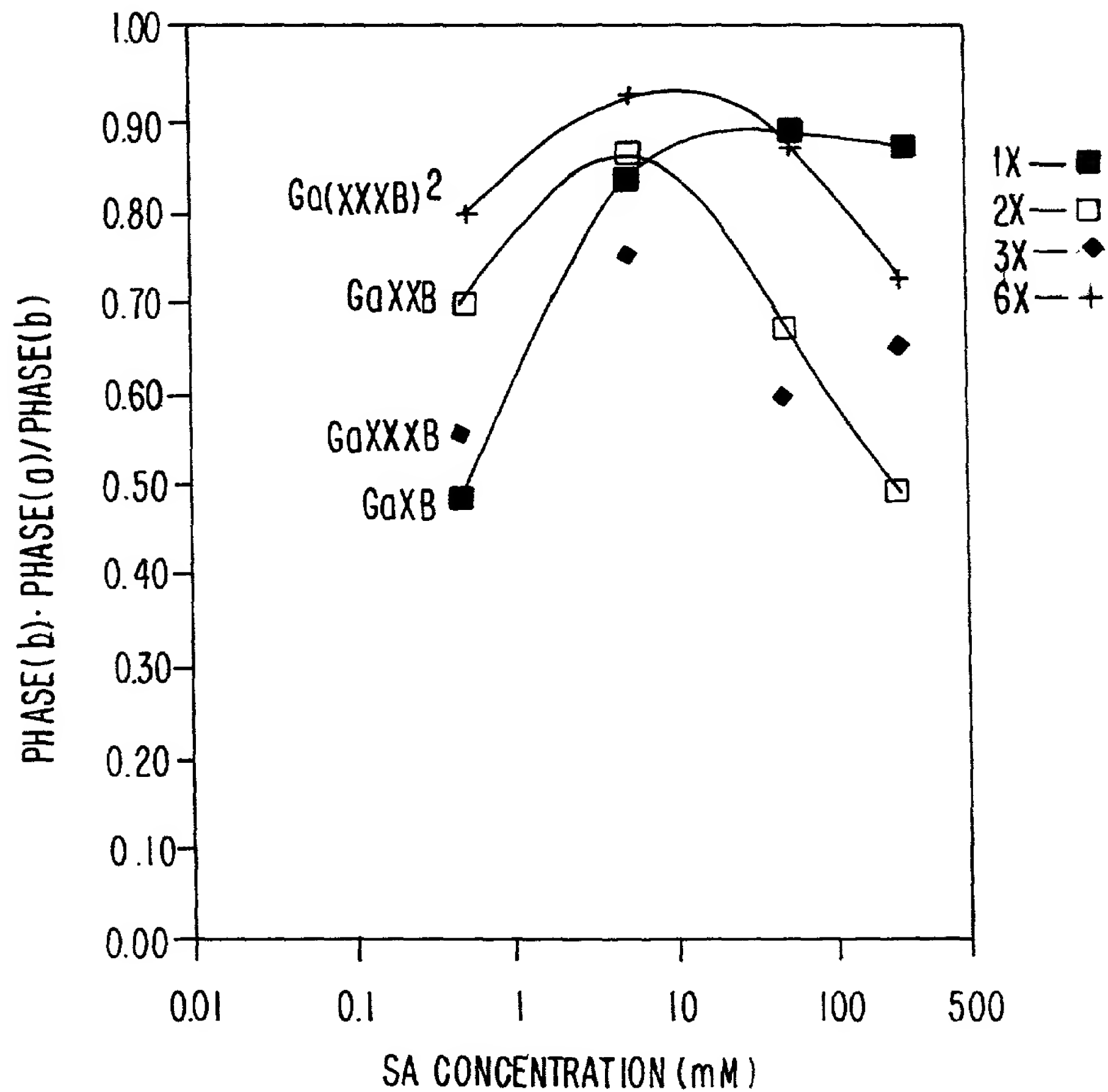


FIG. 12SA TITRATION WITH VARIOUS $GaX..B$ METHOD: 5/4 MEMBRANES

1st LAYERS: 10mM GMPE

1 mM DLP

10 μ M MSLXXB

0.8mM MAAD

0.1 μ M $GaYYSS$

2nd LAYERS: 28 mM GMPE

0.28 μ M $GaX..B$ CHOSEN FROM THE FOLLOWING: $GaXB$ $GaXXB$ $GaXXXB$ $Ga(XXXB)_2$

EXAMPLE OF T₄ ASSAY WITH AMBRIBIOSENSOR

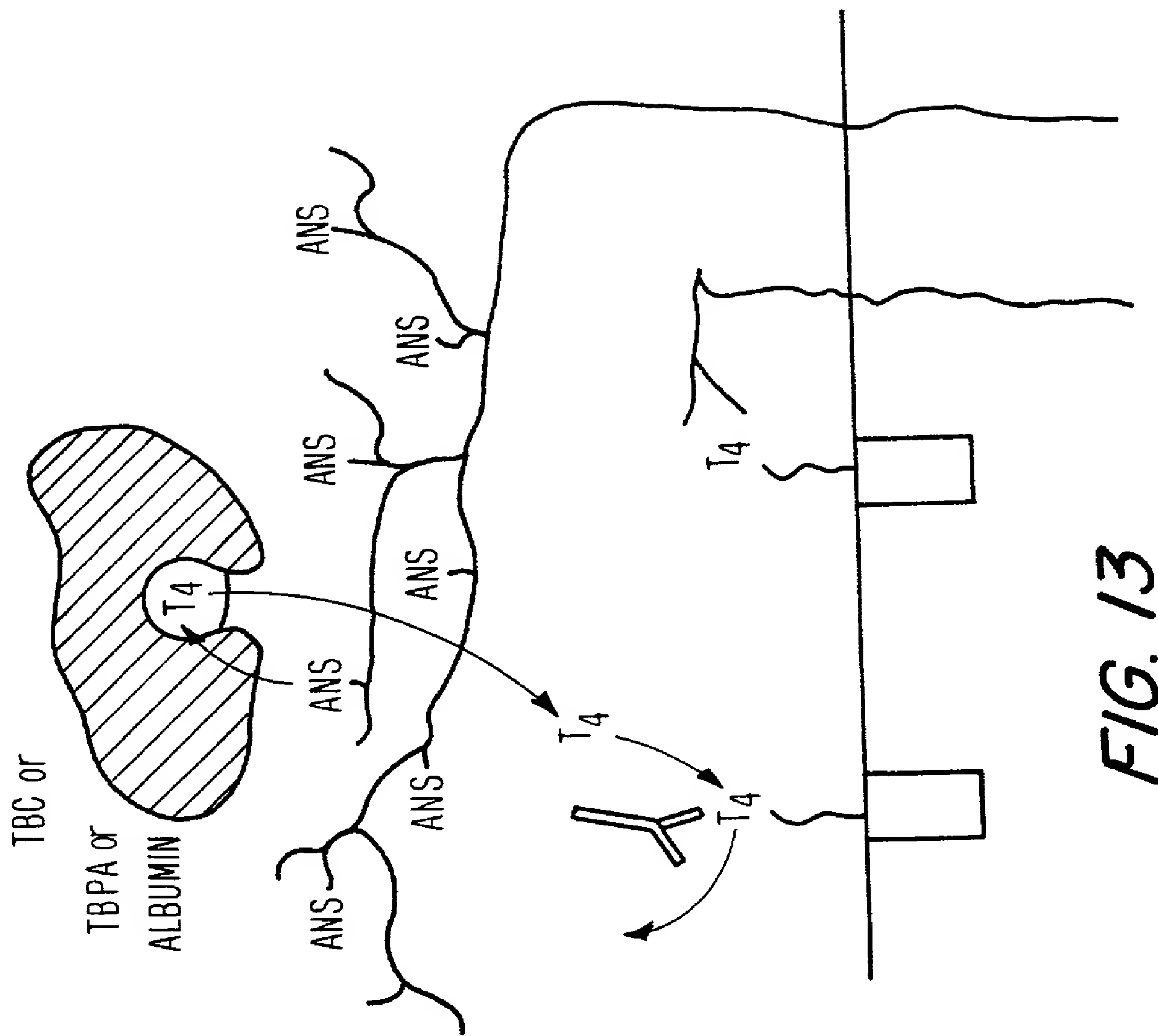
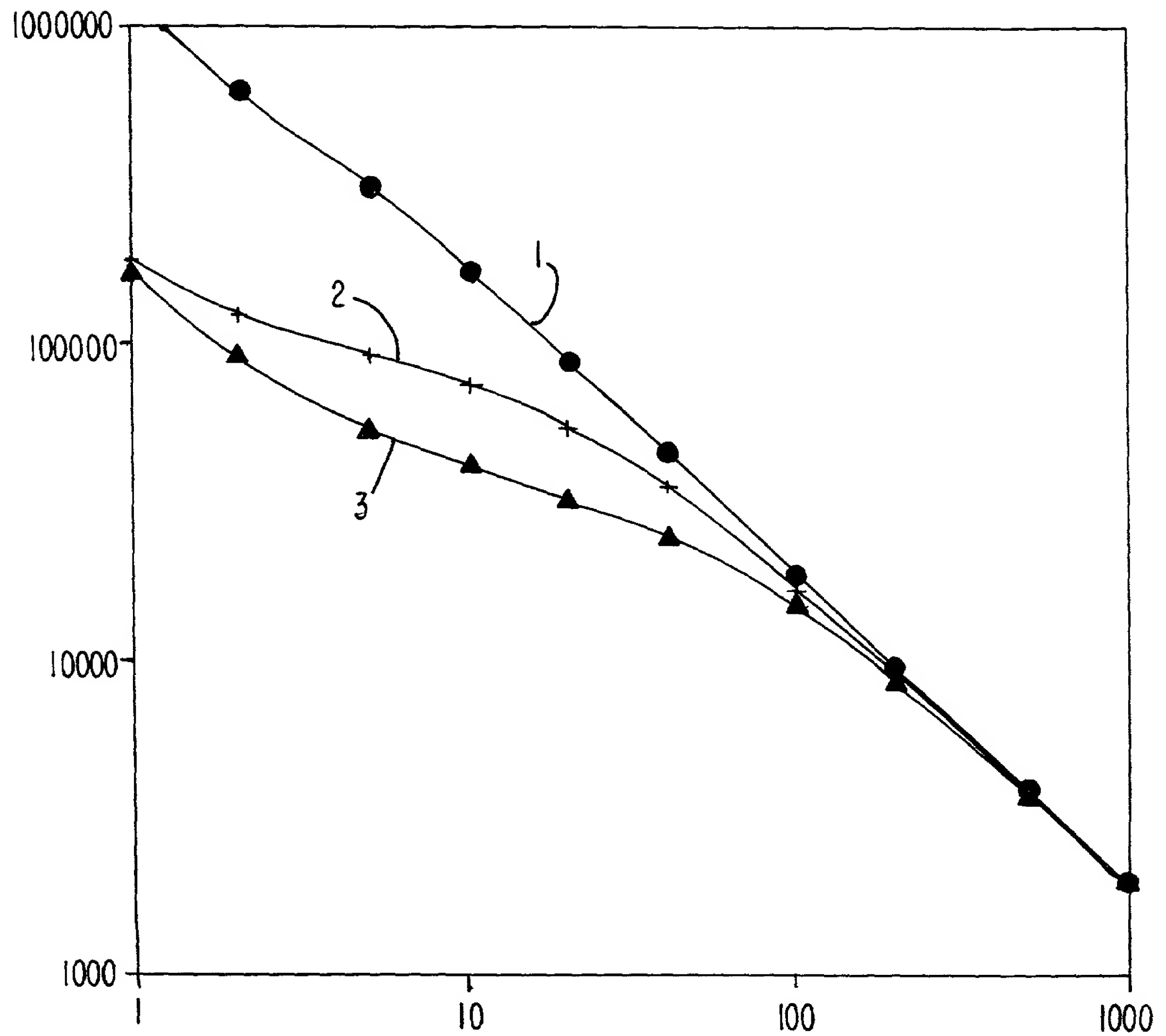
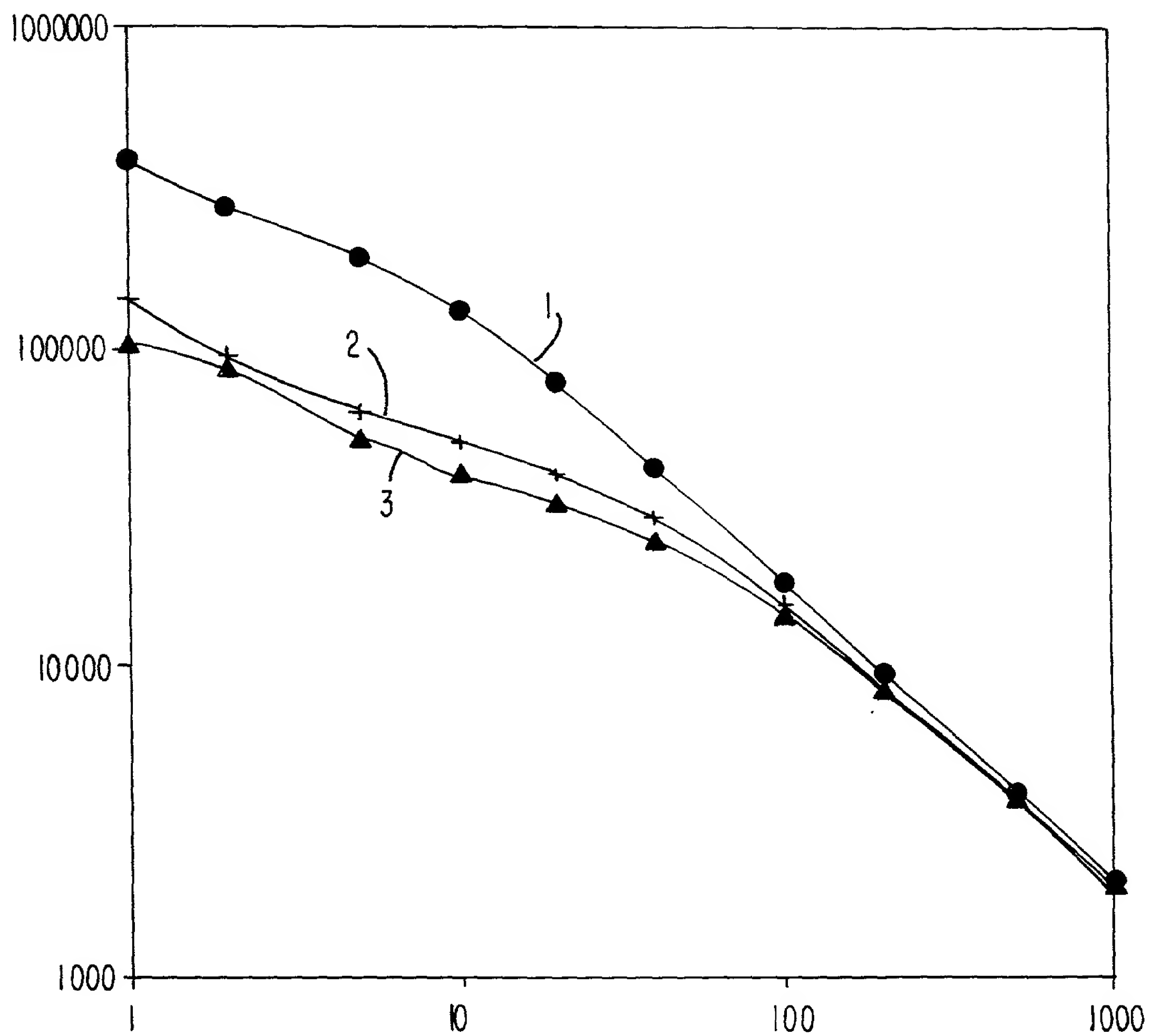


FIG. 13

FIG. 14

- (1) BEFORE THE ADDITION OF GRAMICIDIN/SDS
 (2) AFTER THE ADDITION OF GRAMICIDIN/SDS; STREPTAVIDIN AND BIOTINYLATED FAB'
 (3) AFTER THE ADDITION OF FERRITIN

FIG. 15

- (1) BEFORE ADDITION OF GRAMICIDIN /STREPTAVIDIN
 (2) AFTER ADDITION OF GRAMICIDIN/STREPTAVIDIN AND BIOTINYLATED FAB'S
 (3) AFTER ADDITION OF TSH

Declaration and Power of Attorney

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled SELF ASSEMBLY OF SENSOR MEMBRANES

the specification of which
(check one) is attached hereto. was filed on _____ as United States Application Serial No. or
PCI International Application No. _____ and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefit under Title 35, United States Code, §119 (a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

| Prior Foreign Application(s) | Priority claimed |
|------------------------------|-------------------------------------|
| PN3669 (Number) | Australia (Country) |
| | 20 June 1995 (Day/month/year filed) |
| | Yes No |
| PCT/AU96/00369 (Number) | PCT (Country) |
| | 20 June 1995 (Day/month/year filed) |
| | Yes No |

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below:

| (Application No.) | (Filing Date) |
|-------------------|---------------|
| (Application No.) | (Filing Date) |

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

| (Application No.) | (Filing date) | (Status - patented, pending, abandoned) |
|-------------------|---------------|---|
| (Application No.) | (Filing date) | (Status - patented, pending, abandoned) |

And I hereby appoint

| | |
|--------------------------------------|-------------------------------------|
| George Gottlieb (Reg.No. 22,035) | Any D. Goldsmith (Reg. No. 33,700) |
| Michael I. Rackman (Reg.No. 20,639) | Norbert P. Holler (Reg. No. 17,816) |
| James Reisman (Reg.No. 22,007) | Michael R. Gilman (Reg. No. 34,826) |
| Barry A. Cooper (Reg.No. 25,204) | Tiberlu Weisz (Reg. No. 29,876) |
| David S. Kashman (Reg.No. 28,725) | Jeffrey M. Kaden (Reg. No. 31,268) |
| Allen I. Rubenstein (Reg.No. 27,673) | |

whose address is c/o Gottlieb, Rackman & Reisman, P.C., 270 Madison Avenue, New York NY 10016 (telephone (212) 684-3900), jointly and severally, as my attorneys and/or agents, with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

Direct all correspondence and telephone calls to: Allen I. Rubenstein, Esq. at the address and telephone number shown above.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor Burkhard Raguse

| | |
|----------------------|---------------------|
| Inventor's Signature | Date |
| Residence | St. Ives, Australia |
| Citizenship | Australia |
| Post Office Address | Same as above |

Full name of second joint inventor Roy Ronald John Pace

| | |
|----------------------|--------------------------------|
| Inventor's Signature | Date |
| Residence | Hornebush, NSW 2140, Australia |
| Citizenship | Australia |
| Post Office Address | Same as above |

Full name of third joint inventor, if any Lancel George King

| | |
|----------------------|---------------------|
| Inventor's Signature | Date |
| Residence | Morfield, Australia |
| Citizenship | Australia |
| Post Office Address | Same as above |

Declaration and Power of Attorney

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled SELF ASSEMBLY OF SENSOR MEMBRANES

the specification of which
(check one) is attached hereto. was filed on _____, as United States Application Serial No. or PCT International Application No. _____ and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 (a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)

Priority claimed

| | | | | |
|----------|-----------|----------------------|-----|----|
| PN3669 | Australia | 20 June 1995 | | |
| (Number) | (Country) | Day/month/year filed | Yes | No |

| | | | | |
|----------------|-----------|----------------------|-----|----|
| PCT/AU96/00369 | PCT | 20 June 1996 | | |
| (Number) | (Country) | Day/month/year filed | Yes | No |

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below:

| | |
|-------------------|---------------|
| (Application No.) | (Filing Date) |
|-------------------|---------------|

| | |
|-------------------|---------------|
| (Application No.) | (Filing Date) |
|-------------------|---------------|

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

| | | |
|-------------------|---------------|---|
| (Application No.) | (Filing date) | (Status - patented, pending, abandoned) |
|-------------------|---------------|---|

| | | |
|-------------------|---------------|---|
| (Application No.) | (Filing date) | (Status - patented, pending, abandoned) |
|-------------------|---------------|---|

| | | |
|----------------------|--------------------------------------|-------------------------------------|
| And I hereby appoint | George Gottlieb (Reg.No. 22,035) | Amy D. Goldsmith (Reg. No. 33,700) |
| | Michael I. Rackman (Reg.No. 20,639) | Norbert P. Holler (Reg. No. 17,816) |
| | James Reisman (Reg.No. 22,007) | Michael R. Gilman (Reg. No. 34,826) |
| | Barry A. Cooper (Reg.No. 25,204) | Tiberlu Weiss (Reg. No. 29,876) |
| | David S. Kashman (Reg.No. 28,725) | Jeffrey M. Kaden (Reg. No. 31,268) |
| | Allen I. Rubenstein (Reg.No. 27,673) | |

whose address is c/o Gottlieb, Rackman & Reisman, P.C., 270 Madison Avenue, New York NY 10016 (telephone (212) 684-3900), jointly and severally, as my attorneys and/or agents, with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor Burkhard Raguse

| | | | |
|----------------------|----------------------------|------|----------------|
| Inventor's Signature | <u>B. Raguse</u> | Date | <u>1/10/96</u> |
| Residence | <u>St. Ives, Australia</u> | | |
| Citizenship | <u>Australia</u> | | |
| Post Office Address | <u>Same as above</u> | | |

Full name of second joint inventor, if any Ronald John Pace

| | | | |
|----------------------|---------------------------------------|------|--|
| Inventor's Signature | | Date | |
| Residence | <u>Hornebush, NSW 2140, Australia</u> | | |
| Citizenship | <u>Australia</u> | | |
| Post Office Address | <u>Same as above</u> | | |

Full name of third joint inventor, if any Lionel George King

| | | | |
|----------------------|-----------------------------|------|----------------|
| Inventor's Signature | <u>L. King</u> | Date | <u>1/10/96</u> |
| Residence | <u>Marsfield, Australia</u> | | |
| Citizenship | <u>Australia</u> | | |
| Post Office Address | <u>Same as above</u> | | |

DECLARATION AND POWER OF ATTORNEY
(Cont'd.)

Full name of fourth inventor Violeta Lucija Braach-Maksytis

Inventor's signature *Violeta Braach-Maksytis*

Residence Dulwich Hill, Australia

Citizenship Australia

Post Office Address Same as above

Full name of fifth inventor Bruce Cornell

Inventor's signature *Bruce Cornell*

Residence Neutral Bay, NSW, Australia

Citizenship Australian

Post Office Address same as above.

66/079/693266